Platelet Activation by fMLP-Stimulated Polymorphonuclear Leukocytes: The Activity of Cathepsin G Is Not Prevented by Antiproteases

By Virgilio Evangelista, Grazyna Rajtar, Giovanni de Gaetano, James G. White, and Chiara Cerletti

Human polymorphonuclear leukocytes (PMN) activated by fMLP (in the presence of CaCl₂, fibrinogen, and cytochalasin B) were able to induce aggregation, cytoplasmic Ca²⁺ increase, and thromboxane A₂ production in coinoculated autologous platelets. Cell-free supernatants prepared from formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated PMN were able also to induce platelet activation. Antibodies against cathepsin G and different serin protease inhibitors completely suppressed the activity of PMN-derived supernatants, indicating that cathepsin G is the major platelet activator released by PMN in our system. However, antiproteases only partially affected platelet activation induced by PMN in mixed cell suspensions. Superoxide dismutase and catalase added to the cell suspension did not affect platelet activation nor potentiated serin protease inhibitors, making a role for short-lived oxygen radicals in our experimental system unlikely. Electron microscopic observation of stirred supernatant activity. As the latter activity was dependent on pretreatment of the PMN with cytochalasin B, a substance that enhances secretion of granule-bound material to the cell exterior, it was suggested that some lysosomal enzyme would be the mediator of platelet stimulation. This activity, moreover, was completely inhibited by soybean trypsin inhibitor. Cathepsin G and elastase, two neutral serine proteases contained in the azurophilic granules of PMN, had previously been shown to stimulate platelet function.

In vitro studies of elastase-mediated proteolysis by PMN showed that enzymes released from activated PMN can degrade susceptible substrates in the presence of antiproteases, thereby raising the possibility that PMN-associated proteases are more resistant than are the free cell exterior. It was suggested that some lysosomal enzyme would be the mediator of platelet stimulation. This activity, moreover, was completely inhibited by soybean trypsin inhibitor. Cathepsin G and elastase, two neutral serine proteases contained in the azurophilic granules of PMN, had previously been shown to stimulate platelet function.

Mixed cell suspensions preincubated for 2 minutes at 37°C before stimulation showed a close PMN-platelets contact without any morphologic or biochemical event suggesting platelet activation. Preincubation of the cells without stirring to minimize PMN-platelet interaction before stimulation did not modify subsequent aggregation and platelet cytoplasmic Ca²⁺ increase in control samples. However, in this condition trypsin inhibitor from soybean completely prevented PMN-induced platelet activation. In samples preincubated without stirring in the presence of the antiprotease, activated PMN stuck together but platelets preserved their discoid shape and did not appear significantly activated. We propose that membrane-to-membrane contact could create a microenvironment in which cathepsin G, discharged from stimulated PMN on adherent platelets, is protected from antiproteases.

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enzymes.\(^{6,20}\) We examined whether this possibility also applied to protease-mediated platelet activation by fMLP-stimulated PMN. If so, this phenomenon could also occur in circulating blood, which is replete with a spectrum of proteinase inhibitors.\(^{21}\) Therefore, we tested the effect of different antiprotease on platelet activation induced by PMN-derived supernatant or by PMN in mixed cell suspensions.

**MATERIALS AND METHODS**

**Chemicals.** Superoxide dismutase, catalase, cytochrome C, fMLP, phenolphthalein, phenolphthalein glucuronate acid, pros-taglandin E, (PGE), cytochalasin B, N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES), ethylene glycol-bis (b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), α1-antitrypsin, and soybean trypsin inhibitor (1 mg protein inhibiting 3 to 5 mg trypsin and chymotrypsin with activities of approximately 10,000 BAEE and 40 BTIE units/mg protein, respectively) were purchased from Sigma Chemical Co (St Louis, MO); the specific proteinase inhibitor for cathepsin G and elastase, eglin C (recombinant CGP 32968) was kindly provided by Ciba Geigy (Basel, Switzerland); antihuman cathepsin G and antihuman elastase antibodies were from ICN Immunobiologics (Lisle, IL); cathepsin G purified from human PMN was from Calbiochem (San Diego, CA); egg-white lysozyme and Micrococcus luteus were purchased from Boehringer Mannheim (Mannheim, Germany); Dextran T 500 and Ficoll Hypaque from Pharmacia Fine Chemicals (Uppsala, Sweden); aequorin from ICN Immunobiologicals (Lisle, IL); purified human fibrinogen from Kabi Diagnostica (Milano, Italy); N'-Zethanesulfonic acid (HEPES), ethylene glycol-bis (b-aminoethyl)-N,N,N',N'-tetraacetic acid (EGTA), α1-antitrypsin, and lysozyme were similar in supernatants from PMN and a mixed cell suspension challenged with 1 μmol/L of fMLP. β-glucuronidase activity was detectable in platelets. PMN enzymatic release was not modified by the presence of platelets. β-glucuronidase and lysozyme activity were similar in supernatants from PMN and a mixed cell suspension challenged with 1 μmol/L of fMLP. β-glucuronidase activity was 38.6 ± 3.1 and 38.8 ± 3.5 μg of substrate cleaved/mL of supernatants from PMN and PMN-platelet suspensions stimulated with 1 μmol/L of fMLP, respectively. Lysozyme released was 23.1 ± 4.3 and 20.8 ± 2.3 μg/mL, respectively, in supernatants from PMN and PMN-platelet suspensions.

**Measurement of aggregation and platelet cytoplasmic Ca\(^{2+}\) increase.**

Aggregation was expressed as increase in light transmission 3 minutes after addition of the stimulus. Aggregation reached 10.5 ± 0.5 cm in mixed cell suspensions challenged with 1 μmol/L of fMLP, and 13.7 ± 0.5 cm in platelet suspensions stimulated with PMN-derived supernatants (mean ± SEM of 30 to 40 different experiments) where 16 cm corresponded to 100% light transmission. Calibration of luminescence signals and determination of Ca\(^{2+}\) concentrations were obtained as previously described.\(^{7,10}\) Platelet cytoplasmic Ca\(^{2+}\) increase reached 5.3 ± 0.3 and 4.6 ± 0.4 μmol/L (over Ca\(^{2+}\) resting values of 1.9 ± 0.4 μmol/L in the presence of 1 μmol/L external Ca\(^{2+}\)) in mixed cell suspensions challenged with 1 μmol/L of fMLP and in platelet suspensions stimulated with PMN-derived supernatants, respectively (mean ± SEM of 30 to 40 different experiments). In the case where aggregation and luminescence graphs were directly presented, they represent at least three distinct experiments.

**Table 1. Superoxide Anion Production and Lysosomal Enzyme Release by PMN Activated With fMLP**

<table>
<thead>
<tr>
<th>fMLP (μmol/L)</th>
<th>Cytochrome C Reduction (nmol/10⁶ PMN/40 min)</th>
<th>β-glucuronidase (μg of substrate/mL)</th>
<th>Lysozyme (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>0.2 ± 0.1</td>
<td>2.3 ± 0.9</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>10⁻⁴</td>
<td>0.3 ± 0.1</td>
<td>3.6 ± 1.1</td>
<td>3.5 ± 0.9</td>
</tr>
<tr>
<td>10⁻³</td>
<td>2.4 ± 0.6</td>
<td>18.9 ± 2.7</td>
<td>13.4 ± 2.5</td>
</tr>
<tr>
<td>10⁻²</td>
<td>9.7 ± 1.9</td>
<td>29.1 ± 3.3</td>
<td>21.1 ± 4.1</td>
</tr>
<tr>
<td>10⁻¹</td>
<td>9.8 ± 1.7</td>
<td>39.1 ± 3.1</td>
<td>23.1 ± 4.3</td>
</tr>
</tbody>
</table>

Figures are means ± SE (n = 10). At the highest fMLP concentration used (10⁻⁴ mol/L) β-glucuronidase and lysozyme release were 44.3% ± 2.9% and 68.3% ± 3.2% of total enzyme content, respectively. The total enzyme activities were obtained by solubilizing the cells in Triton X-100.
Fig 1. Electron microscopy of platelet/PMN aggregates (A) and of platelets activated by PMN supernatants (B). PMN alone or in mixed suspensions with platelets were stimulated with 1 μmol/L fMLP. Original magnifications (A) ×9,600 and (B) ×15,600. For further details see Materials and Methods.
reported as nanograms per milliliter of incubate. TxB₂ production reached 23.8 ± 2.6 and 7.9 ± 1.4 ng/mL in mixed cell suspensions challenged with 1 μmol/L of fMLP and in platelet suspension stimulated with PMN-derived supernatants, respectively (mean ± SEM of 30 to 40 different experiments).

Experiments with PMN supernatants. To study the effect of PMN-released products on platelet activation, 800 μL of supernatants (20-second spin in an Eppendorf centrifuge [Leitz, Milano, Italy]) from PMN (0.5 x 10⁸/800 μL) stimulated for 1 minute with fMLP (10⁻⁸ mol/L) were mixed with platelets in the aggregometer and aggregation and luminescence signals were recorded. Antibodies anti-cathepsin G, anti-elastase, α1-antitrypsin, and trypsin inhibitory were tested in samples preincubated with or without stirring. In the latter condition the stirrer was switched on immediately before addition of the stimulus. p-glucuronidase and lysozyme release was similar in both conditions of stirring in the presence and in the absence of soybean trypsin inhibitor (not shown).

Electron microscopy of platelet-PMN aggregates. Electron microscopic observation of aggregates from mixed cell suspension showed both cell types closely intermingled: the platelets were associated already with each other, as well as with PMN. Platelets and PMN appeared in an advanced stage of interaction with a very close contact between membrane of both cell types (Fig 1A). Figure 1B shows aggregates from a platelet sample stimulated by PMN-derived supernatants. Many of the platelets had lost their granules and were in an advanced stage of shape change, secretion, and aggregation.

Measurement of cathepsin G enzymatic activity. Cathepsin G measured in PMN suspensions activated by 1 μmol/L fMLP for 1 minute averaged 0.18 μmol/L (ranges 0.15 to 0.22; n = 5). Cathepsin G enzymatic activity recovered in mixed cell suspensions preincubated with or without stirring was 54.4% ± 6.2% and 48.0% ± 1.2% of the activity measured in PMN stimulated alone, respectively. Because azurophilic granule release, measured as β-glucuronidase, was in contrast not affected by the presence of platelets, the
Platelet activation by PMN supernatants and purified cathepsin G. The platelet-activating material present in PMN supernatants was heat labile (5 minutes at 90°C) and suppressed by preincubation with an antibody against cathepsin G, while it was unaffected by an anti-elastase monoclonal antibody (Fig 2). Purified cathepsin G from human PMN (0.2 μmol/L) induced platelet activation at a similar extent as PMN supernatants. This activity, too, was completely prevented by the anti-cathepsin G antibody (Fig 2).
Purified cathepsin G added to mixed cell suspension induced platelet activation to a similar extent as that observed in fMLP-activated PMN.

Effects of antiproteinases. α1-antitrypsin preincubated with platelet-PMN suspensions completely prevented the effect of exogenous cathepsin G but did not influence platelet activation induced by fMLP-stimulated PMN (Fig 3). Figure 4 shows the effect of α1-antitrypsin (from human plasma) on platelet activation induced by fMLP-stimulated PMN or by PMN-derived supernatants. The effect of PMN-derived supernatants was completely abolished by this antiproteinase (IC₅₀: 130, 140, and 160 μg/mL for aggregation, Ca²⁺ increase, and TxB₂ production, respectively). In contrast, platelet activation in mixed cell suspensions challenged with fMLP appeared only partially affected.

Soybean trypsin inhibitor from soybean completely prevented platelet activation induced by PMN-derived supernatants. This effect was dose-dependent on the three parameters studied (IC₅₀: 1.3, 1.0, and 2.0 μg/mL for aggregation, Ca²⁺ increase, and TxB₂ production, respectively). In contrast, platelet activation induced by PMN in mixed cellular suspensions was partially affected by soybean trypsin inhibitor only at concentrations of at least 10 to 20 times higher than that required to completely prevent supernatant activity (Fig 4). Elgin C, a low molecular weight inhibitor of cathepsin G and elastase, completely blocked platelet stimulating material present in PMN supernatants (IC₅₀: 6.5, 6.5, and 8 μg/mL for aggregation, Ca²⁺ increase, and TxB₂ production, respectively), but only partially inhibited platelet activation in mixed cellular suspensions (Fig 4).

Because hydrogen peroxide and superoxide anion have been shown to stimulate platelet function, the hypothesis that in mixed cellular suspensions short-lived radicals could play a role (either inducing per se platelet activation or potentiating the effect of released serine protease) was also tested. The effect of superoxide dismutase and catalase at concentrations that abolished cytochrome C reduction (both 50 μg/mL) was evaluated in mixed cellular suspensions activated by 1 μmol/L fMLP, in the presence or in the absence of soybean trypsin inhibitor (100 μg/mL). Results of these experiments oxygen radical scavengers neither affected per se platelet activation nor significantly modified the activity of soybean trypsin inhibitor (Fig 5).

Electron microscopic examination of stirred or unstirred platelet-PMN samples. Electron microscopic examination of stirred PMN-platelet suspensions preincubated for 2 minutes at 37°C before stimulation showed a close PMN-platelet contact with the formation of small clumps in which platelets appeared to contact to PMN membranes without any morphologic or biochemical event suggesting platelet activation. Figure 6A demonstrates several platelets having a discoid configuration in close proximity to neutrophils. In contrast, cells from an unstirred sample appeared dispersed and no platelet-PMN interaction was observed (Fig 6B).

Effect of platelet-PMN preincubation on efficiency of antiproteinases. To verify if PMN-platelet contact possibly occurring during preincubation with stirring may protect released cathepsin G from antiproteinases, the efficacy of these inhibitors on platelet activation induced by PMN was tested in mixed cell suspensions preincubated without stirring. Under these conditions subsequent aggregation and platelet cytoplasmic Ca²⁺ increase in control samples were not modified, but soybean trypsin inhibitor completely blocked platelet activation induced by PMN (Fig 7). On the other hand α1-antitrypsin at a concentration (200 μg/mL) that did not significantly affect aggregation or platelet cytoplasmic Ca²⁺ increase under stirring conditions (15.5% ± 7.6% and 16.6% ± 2.5% inhibition), induced 50.3% ± 7.6% and 62.2% ± 7.7% inhibition (means ± SEM; n = 4) of aggregation and platelet cytoplasmic Ca²⁺ increase when cells were preincubated without stirring. Figure 8 shows the morphology of the PMN-platelet suspension preincubated without stirring for 2 minutes at 37°C with soybean trypsin inhibitor (100 μg/mL) before stimulation with 1 μmol/L of
Fig 6. Electron microscopy of unstimulated platelet/PMN suspensions incubated for 2 minutes at 37°C with stirring (1,000 rpm) (A) or without stirring (B). Original magnifications × 9,600.
In accordance with the biochemical findings, platelets preserved their discoid shape and did not appear to have been significantly activated even when they were in close association with PMN. PMN adhered to each other, while platelets appeared to resist the stimulation. In corresponding samples preincubated and stirred with the same concentration of soybean trypsin inhibitor, platelet and PMN were aggregated and both cells showed marked changes associated with activation, similar to the activated control sample (see Fig 1).

DISCUSSION

In this study, the prominent role of cathepsin G released by fMLP-stimulated PMN as a platelet activator was confirmed, as shown by the inhibitory efficacy of specific antibodies. Previous reports had demonstrated that cathepsin G and elastase, two serine proteases, released by activated PMN stimulate platelet function. In particular, cathepsin G was able to induce platelet aggregation, cytoplasmic calcium increase, and serotonin release, acting as a strong platelet agonist. Its activity was independent of amplification mechanisms such as ADP release or TxA2 production. Recently, Selak and Smith have identified a specific receptor for cathepsin G on the platelet surface, suggesting a role for this serine protease in platelet function.
Our results suggest that cathepsin G released in PMN-platelet suspensions is protected from different inhibitors. In fact, while antiproteinases such as α1-antitrypsin, soybean trypsin inhibitor, and elgin C all prevented platelet activation induced by purified cathepsin G or by PMN supernatants separated from the cells, they were much less effective when platelets were activated by stimulated PMN in mixed cell suspensions.

A similar phenomenon related to the action of elastase released by activated PMN has been reported. The presence of PMN and platelets may protect released cathepsin G from various inhibitors, by at least two mechanisms: (1) inactivation of antiproteinases through oxidation mediated by free radicals released by PMN during respiratory burst activity, and (2) close contact between PMN and platelets that physically impede the antiproteinases to reach cathepsin G secreted in a protected microenvironment.

The results obtained with soybean trypsin inhibitor, which is resistant to oxidation, indicate that short-lived oxygen radicals released by PMN in our system do not play an essential role in the described protection phenomenon. Weitz et al. also found that oxidative inactivation of plasma proteinase inhibitors does not explain the failure of soybean trypsin inhibitor to prevent elastase-mediated fibrinogenolysis.

In addition, platelet-activating activity of oxygen radicals does not seem to play a role in mixed cell suspensions, because superoxide dismutase (SOD) and catalase did not affect per se PMN-induced platelet activation nor potentiated the inhibitory effect of soybean trypsin inhibitor.

The second mechanism seems more likely, because a close contact between PMN and platelets occurs when cells were incubated under stirring conditions before addition of the stimulus, as shown by electron microscopy. Moreover, the protective effect was no more evident when the samples were not stirred during preincubation as a means to minimize cell-cell contact. Results from these experiments also showed that preincubation without stirring did not reduce the subsequent aggregation and platelet cytoplasmic Ca2+ increase that occurred when control samples were stirred upon addition of FMLP. In mixed cell suspensions preincubated with soybean trypsin inhibitor without stirring, platelet activation induced by PMN was totally suppressed. The same concentration of the inhibitor was only partially effective in prestirred samples (Fig 7).

In parallel with biochemical parameters, electron microscopy of previously unstirred samples showed that platelets were not activated and had retained their discoid configuration. They were physically close to PMN, but the degree of interaction was very limited. Even so, PMN adhered each other, while platelets appeared to resist the stimulation. PMN and platelets looked simply mixed together with little cell-cell interaction going on. On the other hand, when samples containing soybean trypsin inhibitor were stirred before stimulation, PMN and platelets appeared to be aggregated, both cells demonstrating marked changes associated with activation.

These data are in agreement with the hypothesis that the close PMN-platelet contact occurring during preincubation of stirred samples before addition of the stimulus would create a microenvironment in which released cathepsin G accumulates at relatively high concentrations and is protected from inhibitors. This view is reinforced by the observation that the addition of purified cathepsin G to prestirred mixed cell suspensions induced full platelet activation, which was effectively prevented by antiproteinase inhibitors. In this case it is reasonable to suggest that exogenous cathepsin G was homogeneously distributed throughout the sample and was therefore rapidly inactivated by antiproteinases.

The mechanism(s) of platelet-PMN interaction in our system remain(s) to be elucidated. Mechanical stirring of the sample may induce some platelet stimulation leading to the adhesion of these cells to PMN, as shown for thrombin-activated platelets. Whatever the molecular mechanism of platelet-PMN interaction in our system, the protective phenomenon described for the first time in this paper suggests an interesting functional role for PMN-platelet adhesion.

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