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

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Human polymorphonuclear leukocytes (PMN) activated by fMLP (in the presence of $CaCl_2$, fibrinogen, and cytochalasin B) were able to induce aggregation, cytoplasmic Ca^{2+} increase, and thromboxane A_2 production in coincubated autologous platelets. Cell-free supernatants prepared from n-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, antiproteinases 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 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 Ca^{2+} 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 antiproteinase, activated PMN sticked 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 antiproteinases.

A NUMBER OF epidemiologic studies have shown a positive correlation between white blood cell counts and risk of ischemic disease.[1] Experimental studies in animals also suggest that polymorphonuclear leukocytes (PMN) play an important role in the pathophysiology of myocardial infarction. Accumulation of PMN at the site of myocardial ischemia may exacerbate tissue injury through the release of oxygen radicals and proteolytic enzymes.[2]

Furthermore, an increased functional activity of PMN in vitro from patients with stable angina has been demonstrated. In vivo activation of PMN in patients with unstable angina and myocardial infarction is manifested by increased plasma levels of a peptide from the fibrinogen Bβ chain, a specific product of fibrin(ogen) degradation by neutrophil elastase. PMN activation in unstable angina suggests that patients who will develop acute myocardial ischemia may have abnormal leukocyte function before the onset of an acute event.[3] Recently, several studies have tried to shed more light on the mechanisms by which PMN could contribute to vascular occlusion.

Platelet activation induced by stimulated PMN has, in fact, been demonstrated in animals and in humans.[4-8] Leukocyte-derived products may promote platelet aggregation, serotonin release, and platelet cytoplasmic calcium increase.[9-13]

In a previous study PMN challenged with different agonists induced platelet activation, measured as platelet aggregation, cytoplasmic calcium increase, and thromboxane B_2 (TxB_2) production.[14] BN-52021, a platelet activating factor (PAF) receptor antagonist, did not affect platelet activation in mixed cellular suspensions by PMN challenged with n-formyl-methionyl-leucyl-phenylalanine (fMLP), suggesting that PAF is not involved in this phenomenon. Aspirin, at concentrations completely blocking TxB_2 production, did not influence aggregation or platelet cytoplasmic Ca^{2+} increase (in mixed cell suspensions), suggesting that TxA_2 synthesis was not essential for PMN-induced platelet activation. Activated PMN released platelet activating material in extracellular medium as indicated by PMN-derived 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.[12,13,15]

In vitro studies of elastase-mediated proteolysis by PMN showed that enzymes released from activated PMN can degrade susceptible substrates in the presence of antiproteinases, thereby raising the possibility that PMN-associated proteinases are more resistant than are the free

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enzymes.[16-20] 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 antiproteinase 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 glucuronic acid, prostaglandin E_1 (PGE_1), 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 BTEE 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 Immunobiologicals (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); Triton X-100 was obtained from Aldrich Chimica S.r.l. (Milano, Italy); purified human fibrinogen from Kabi Diagnostica (Stockholm, Sweden); aequorin from Dr J. Blinks (Mayo Clinic, Rochester, MN). Anti-cathepsin G and anti-elastase antibodies obtained as sheep Ig fraction in phosphate-buffered saline (PBS) containing 0.1% NaN_3 and 0.01% thiomersal were dialyzed overnight at 4°C against PBS pH 7.4, stored in aliquots at −20°C, and thawed just before use.

Preparation of PMN and aequorin-loaded platelets. PMN were isolated from citrated human blood as described.[22] PMN were washed and resuspended in HEPES-Tyrode buffer (pH 7.4) containing: 129 mmol/L NaCl, 9.9 mmol/L $NaHCO_3$, 2.8 mmol/L KCl, 0.8 mmol/L KH_2PO_4, 0.8 mmol/L $MgCl_2$-$6H_2O$, 5.6 mmol/L Dextrose, 10 mmol/L HEPES, and 1 mmol/L $CaCl_2$. Cellular suspensions (0.5×10^7 cells/mL) contained 95% PMN; an average of 1 platelet/20 leukocytes was usually observed. Autologous platelets were washed and loaded with aequorin according to Yamaguchi et al.[23] Previous experiments showed that this procedure did not modify platelet function in our experimental condition.

Experimental procedures. Aequorin-loaded platelets (10^8 cells/mL), PMN (0.5×10^7 cells/mL), or a mixture of the two cells (platelets/PMN ratio = 20) were incubated in a final volume of 1 mL in the presence of 1 mmol/L Ca^{2+}, 0.38 mg/mL fibrinogen, and 2.5 μg/mL cytochalasin B in the "Platelet Ionized Calcium Aggregometer" (PICA; Chrono-Log, Mascia Brunelli, Milano, Italy) for 2 minutes at 37°C under constant stirring at 1,000 rpm, unless otherwise specified, with or without α1-antitrypsin, soybean trypsin inhibitor, eglin C, superoxide dismutase, and catalase. fMLP or purified cathepsin G were then added to the cell suspension and aggregation and luminescence were recorded simultaneously on a Dual Channel Strip Chart Recorder (Chrono-Log). Three minutes after stimulus the samples were cooled to 0°C and centrifuged in an Eppendorf centrifuge for 1.5 minutes. Supernatants were collected and stored at −20°C for TxB_2 assay and lysosomal enzyme activity. Cathepsin G activity in PMN and mixed cell suspension was determined by continuous monitoring of the specific chromogenic substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide hydrolysis according to Nakajima et al.[24] Briefly, 5 μL of a 100 mmol/L solution of the specific synthetic substrate in N-methypirrolidinone (final concentration 0.5 mmol/L) was added to the cell suspension before the addition of the stimulus and its cleavage was monitored by following the release of p-nitroanilide at 410 nm, in the supernatant prepared 1 minute after cell stimulation. The activity measured in the samples was then compared with a standard curve obtained by measuring the activity of different concentrations of purified cathepsin G. Lysozyme activity was determined by measuring the lysis of the substrate *M luteus,* at 540 nm and comparing the results with those obtained with known amounts of egg-white lysozyme.[25] β-glucuronidase activity was determined using phenolphthalein glucuronate as substrate, monitoring its cleavage at 540 nm.[25]

Superoxide anion production was determined by measuring the reduction of cytochrome C as described,[26] and expressed as nanomoles of cytochrome C reduced by 10^6 PMN/40 min.

Preliminary experiments showed that in our experimental conditions fMLP (10^{-9} to 10^{-6} mol/L) was able to trigger secretion of both lysozyme and β-glucuronidase and to induce superoxide anions production from PMN (Table 1). No lysozyme and very limited (less than 5 μg substrate cleaved/mL) β-glucuronidase activity were 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 fMLP, respectively. Lyzozyme 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.[27,28] 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 mmol/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

fMLP (mol/L)	Cytochrome C Reduction (nmol/10^6 PMN/40 min)	β-glucuronidase (μg of substrate/mL)	Lysozyme (μg/mL)
—	0.2 ± 0.1	2.3 ± 0.9	2.2 ± 1.1
10^{-9}	0.3 ± 0.1	3.6 ± 1.1	3.5 ± 0.9
10^{-8}	2.4 ± 0.6	18.9 ± 2.7	13.4 ± 2.5
10^{-7}	9.7 ± 1.9	29.1 ± 3.3	21.1 ± 4.1
10^{-6}	9.8 ± 1.7	39.1 ± 3.1	23.1 ± 4.3

Figures are means ± SE (n = 10). At the highest fMLP concentration used (10^{-6} mol/L) β-glucuronidase and lysozyme release were 44.3% ± 2.9% and 58.3% ± 3.2% of total enzyme content, respectively. The total enzyme activities were obtained by solubilizing the cells in Triton X-100.

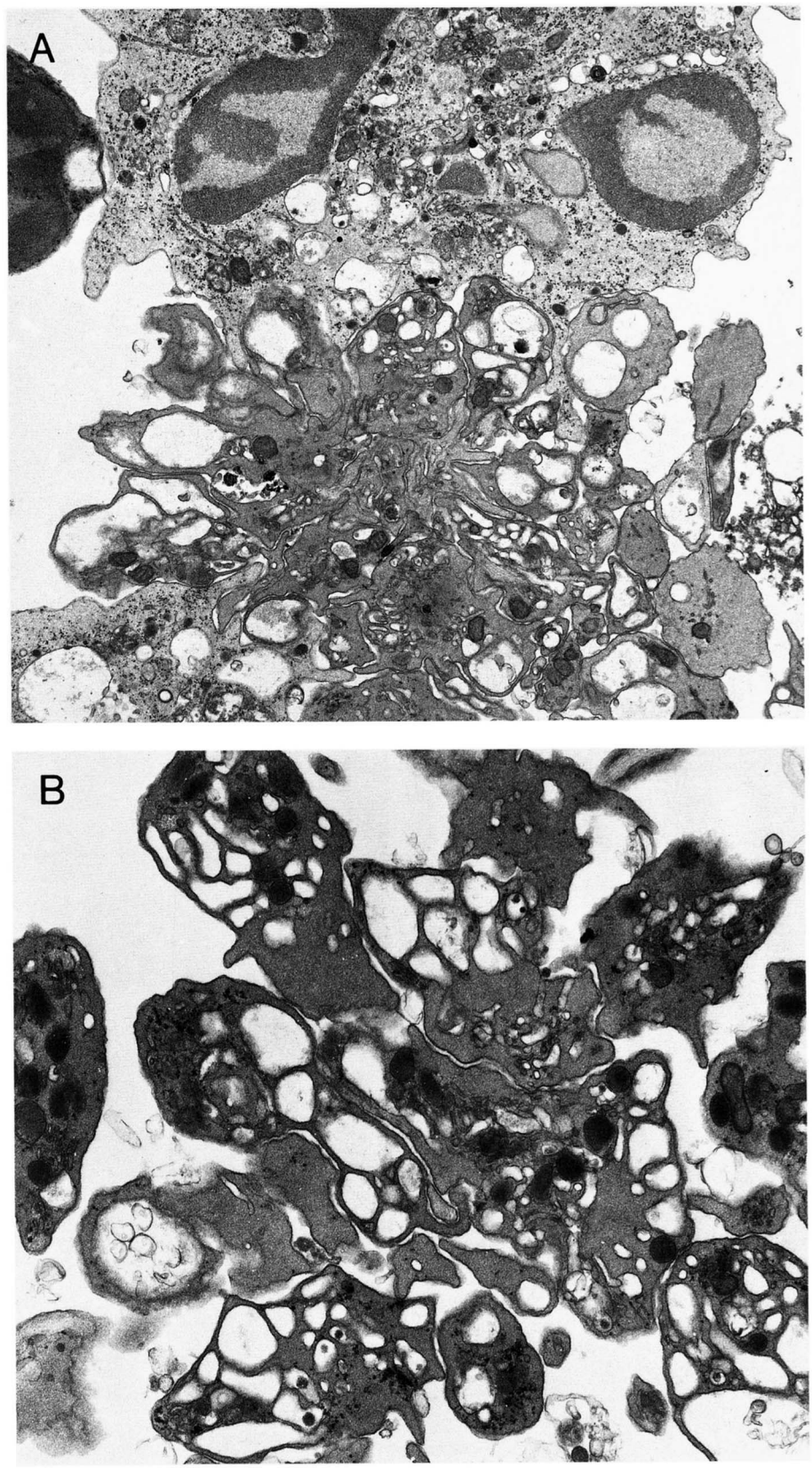

Fig 1. Electron microscopy of platelet/PMN aggregates (A) and of platelets activated by PMN supernatants (B). PMN alone or in mixed suspensions with platelets were stimulated with 1 μmol/L fMLP. Original magnifications (A) ×9,600 and (B) ×15,600. For further details see Materials and Methods.

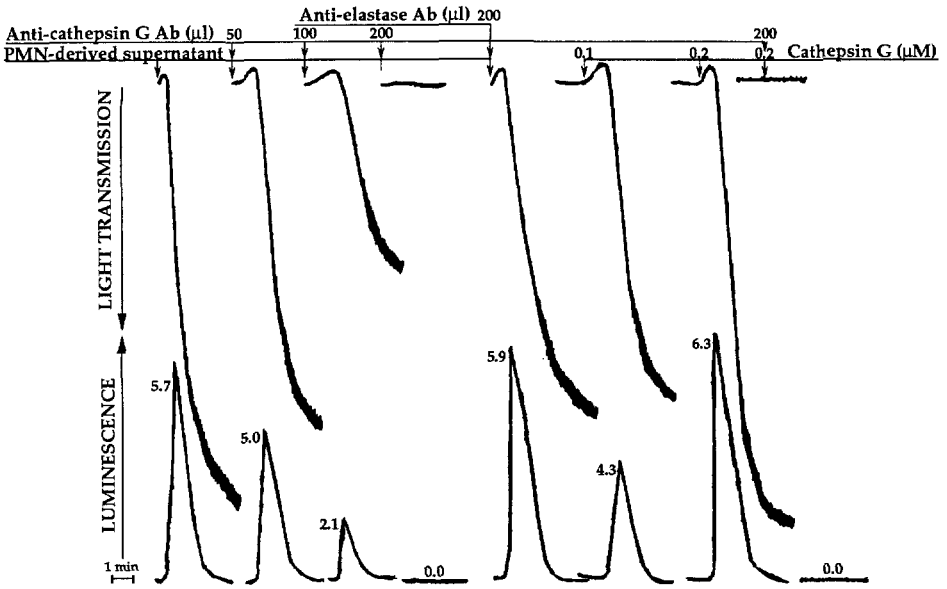

Fig 2. Representative tracings of aggregation response (top) and of luminescence signal (bottom) of aequorin-loaded platelets stimulated by supernatant from PMN activated by 1 μmol/L fMLP or by purified cathepsin G. Effect of different concentrations of anti-cathepsin G or anti-elastase antibodies added to PMN supernatants or to cathepsin G 30 seconds before addition to platelets. Numerical values indicated near the luminescence peaks indicate the increase of platelet cytoplasmic Ca^{2+} (μmol/L). Resting Ca^{2+} value in this experiment was 1.3 μmol/L in the presence of 1 mmol/L external Ca^{2+}.

TxB_2 determination. TxB_2 was quantitated by radioimmunoassay, using a specific antiserum kindly provided by Prof C. Patrono (Catholic University, Rome, Italy), as described.[29] TxB_2 values are reported as nanograms per milliliter of incubate. TxB_2 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 (0.5×10^7/800 μL) stimulated for 1 minute with fMLP (10^{-6} mol/L) were mixed with platelets in the aggregometer and aggregation and luminescence signals were recorded. Antibodies anti-cathepsin G, anti-elastase, α1-antitrypsin, soybean trypsin inhibitor, or eglin C were added to supernatant immediately before addition to platelets. Supernatants from unstimulated PMN were unable to induce any measurable platelet activation.

Experiments with different stirring conditions. We evaluated the possibility that a close membrane-to-membrane contact between PMN and platelet occurring in mixed cell suspension preincubated at 37°C at 1,000 rpm stirring could result in the protection of cathepsin G-induced platelet activation. For this purpose, experiments were performed in which the inhibitory effect of soybean trypsin inhibitory was tested in samples preincubated with or without stirring. In the latter condition the stirrer was switched on immediately before addition of the stimulus. β-glucuronidase and lysozyme release was similar in both conditions of stirring in the presence and in the absence of soybean trypsin inhibitor (not shown).

Morphologic studies. Portions of all control and experimental samples were prepared for study in the electron microscope, as previously reported.[30] Briefly, the samples were mixed with an equal volume of 0.1% glutaraldehyde in White's saline for 15 minutes and sedimented to pellets in a tabletop centrifuge. Supernatant was discarded and the pellet mixed with 3% glutaraldehyde in the same buffer (White's saline). After 1 hour in 3% glutaraldehyde the samples were combined with fresh 3% glutaraldehyde and shipped overnight to another laboratory for further processing. The samples after arrival were fixed in 1% osmic acid in distilled water containing 0.1% potassium ferrocyanide. The samples were then dehydrated in a series of alcohols, embedded in Epan 812, and sectioned on an LKB ultramicrotome. All observations were made in a Philips electron microscope on sections contrasted with uranyl acetate and lead citrate.

RESULTS

As already shown,[14] platelet aggregation, cytoplasmic Ca^{2+} increase, and TxB_2 production were induced by PMN in mixed cellular suspensions challenged with 1 μmol/L fMLP, or by supernatants obtained from PMN 1 minute after stimulation with 1 μmol/L fMLP. This stimulus was unable to induce aggregation, cytoplasmic Ca^{2+} increase, and TxB_2 production in samples containing platelets alone. On the contrary, fMLP induced a limited, but concentration-dependent aggregation of PMN, reaching the maximum (3.0 ± 0.2 cm) at 1 μmol/L of the stimulus. TxB_2 was undetectable in fMLP-stimulated PMN.

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

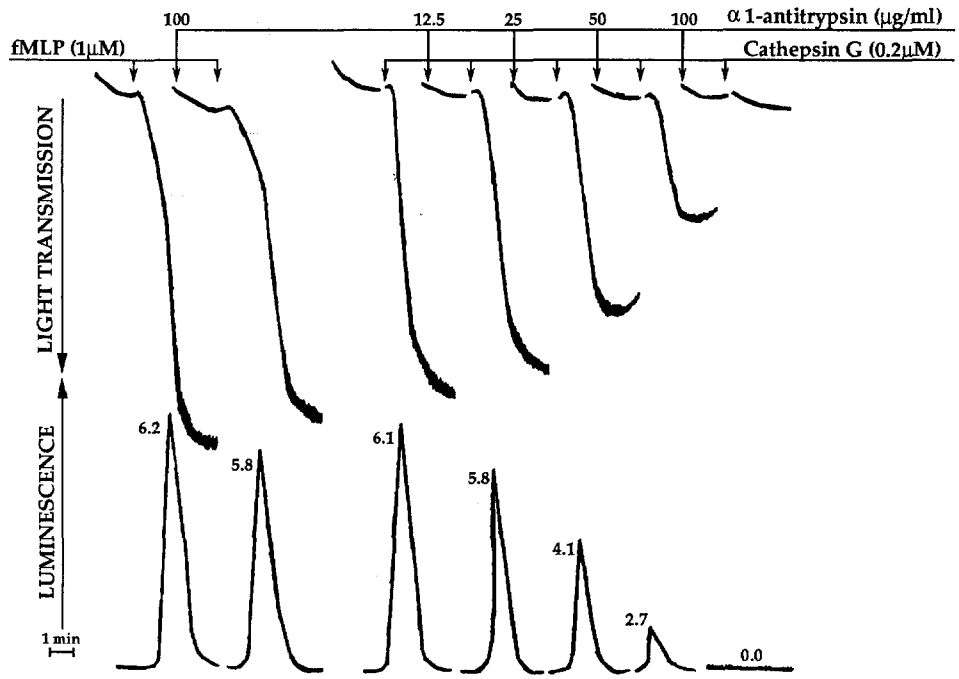

Fig 3. Representative tracings of aggregation response (top) and of luminescence signal (bottom) of mixed suspensions of PMN and aequorin-loaded platelets stimulated by 1 μmol/L fMLP or by purified cathepsin G. Effect of different concentrations of α1-antitrypsin preincubated with the cells suspensions for 2 minutes before addition of the stimulus. Numerical values indicated near the luminescence peaks indicate the increase of platelet cytoplasmic Ca^{2+} (μmol/L). Resting Ca^{2+} value in this experiment was 1.7 μmol/L in the presence of 1 mmol/L external Ca^{2+}.

more likely explanation for this reduced catalytic activity of cathepsin G in mixed cell populations is that the enzyme linked to platelets becomes unavailable to the substrate. This interpretation is further supported by the recent demonstration that cathepsin G has a specific receptor on platelet surface.[31]

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).

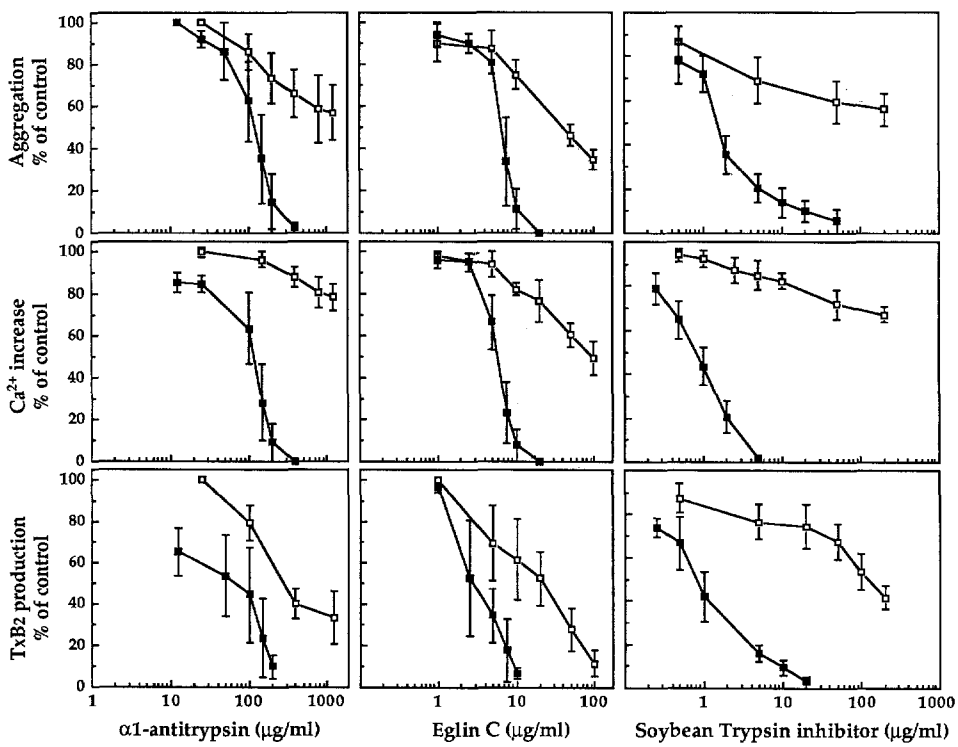

Fig 4. Effect of α1-antitrypsin, eglin C, and soybean trypsin inhibitor on platelet aggregation, intracellular Ca^{2+} increase, and TxB_2 production induced by stimulated PMN (□) or supernatants (■) obtained 1 minute after activation of PMN with 1 μmol/L fMLP. Results are means and SEM of 5, 6, and 17 to 35 experiments, respectively, and are expressed as percent of control values obtained in the absence of inhibitor for each experiment. Control values for each parameter studied are reported in Materials and Methods.

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 (IC_{50}: 130, 140, and 160 μg/mL for aggregation, Ca^{2+} increase, and TxB_2 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 (IC_{50}: 1.3, 1.0, and 2.0 μg/mL for aggregation, Ca^{2+} increase, and TxB_2 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). Eglin C, a low molecular weight inhibitor of cathepsin G and elastase,[32,33] completely blocked platelet stimulating material present in PMN supernatants (IC_{50}: 6.5, 6.5, and 8 μg/mL for aggregation, Ca^{2+} increase, and TxB_2 production, respectively), but only partially inhibited platelet activation in mixed cellular suspensions (Fig 4).

Because hydrogen peroxide[11] and superoxide anion[10] 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). In these experiments oxygen radicals 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 prestirring on efficacy of antiproteinases. To verify if PMN-platelet contact possibly occurring during preincubation with stirring may protect released

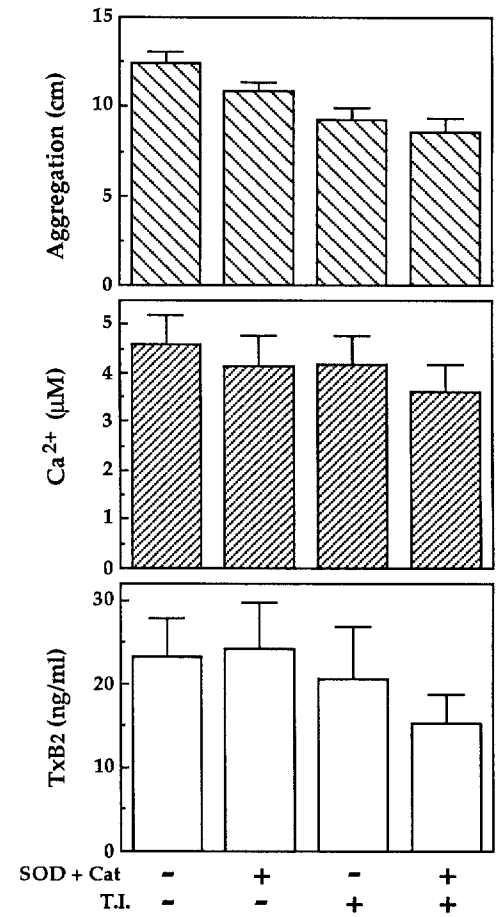

Fig 5. Platelet aggregation, intracellular Ca^{2+} increase and TxB_2 production in mixed cell suspensions activated with 1 μmol/L fMLP. Effect of 2 minutes of preincubation at 37°C with superoxide dismutase (SOD) and catalase (Cat) (both at 50 μg/mL) in the presence and in the absence of soybean trypsin inhibitor (T.I., 100 μg/mL). Results are means and SEM of six experiments. The experimental samples preincubated with SOD + Cat were not significantly different from their appropriate controls (column 2 *v* 1 and column 4 *v* 3; analysis of variance).

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 Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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

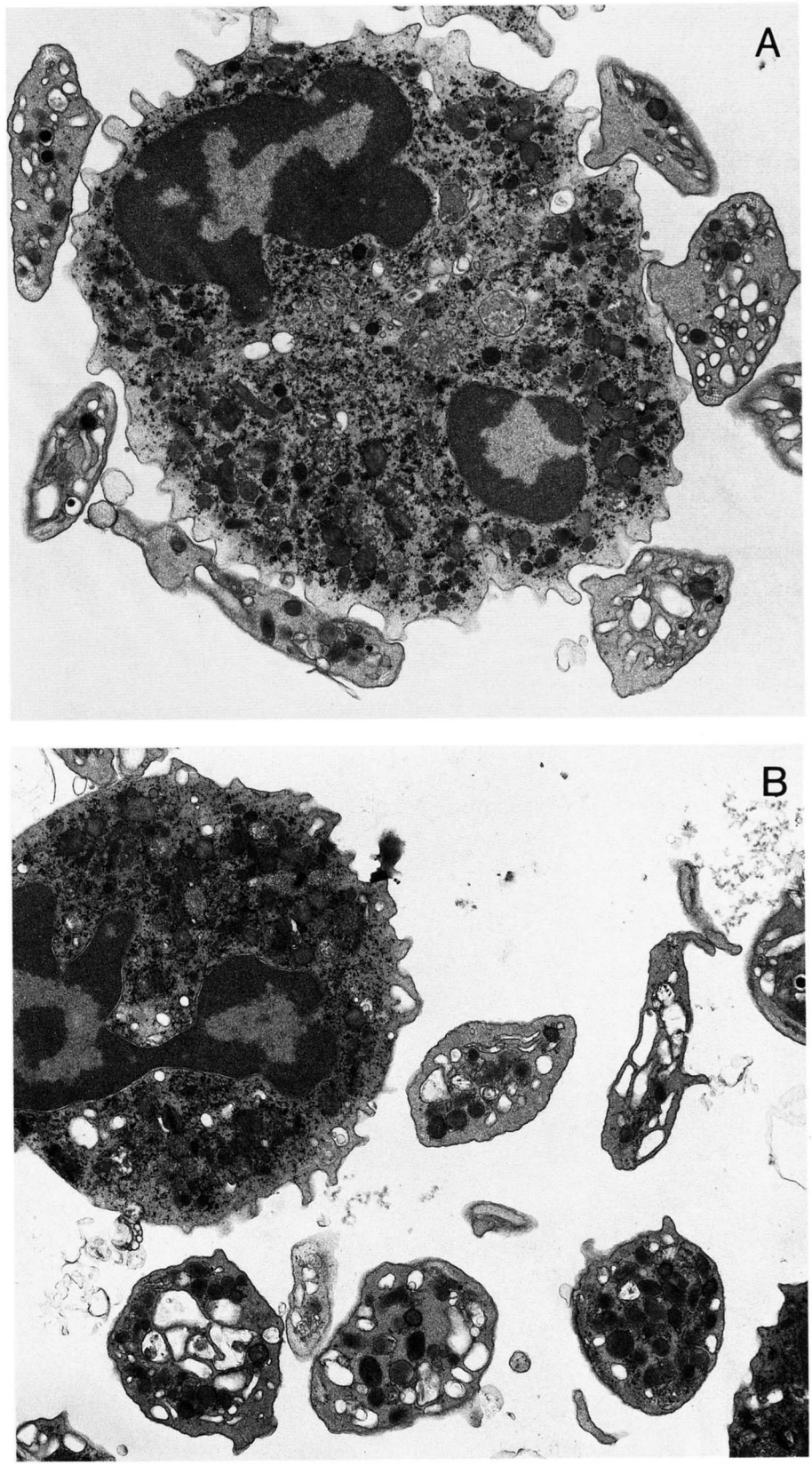

Fig 6. Electron microscopy of unstimulated platelet/PMN suspensions incubated for 2 minutes at 37°C with stirring (1,000 rpm) (A) or without stirring (B). Original magnifications × 9,600.

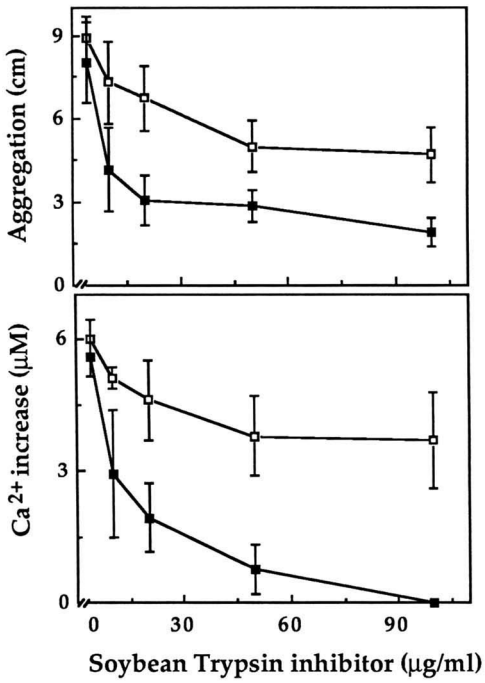

Fig 7. Platelet aggregation and intracellular Ca^{2+} increase induced by 1 μmol/L fMLP in mixed cell suspensions preincubated for 2 minutes at 37°C with stirring at 1,000 rpm (□) or without stirring (■) before addition of the stimulus. Effect of soybean trypsin inhibitor. Results are means and SEM of six experiments.

fMLP. 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.[12,13,15] 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 TxA_2 production.[12] 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.[31]

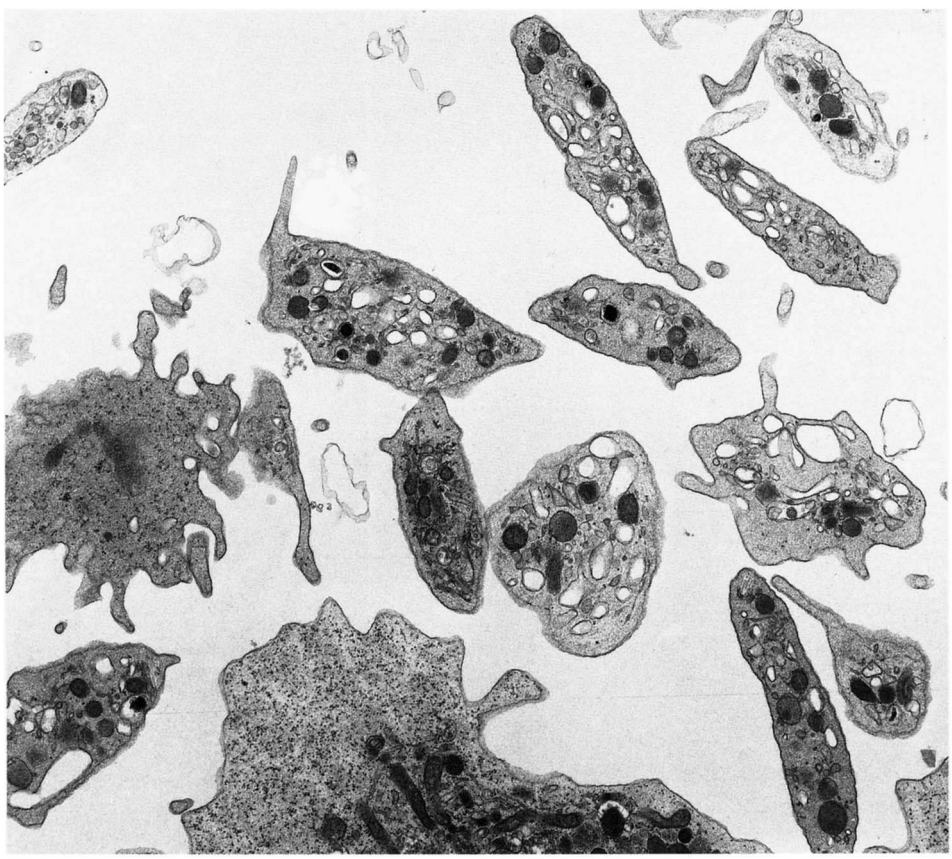

Fig 8. Electron microscopy of platelet/PMN suspensions preincubated in the presence of soybean trypsin inhibitor (100 μg/mL) for 2 minutes at 37°C without stirring and then stimulated with 1 μmol/L fMLP. Original magnification × 9,600.

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 eglin 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.[17,19,20]

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,[34,35] and (2) close contact between PMN and platelets that physically impede the antiproteinases to reach cathepsin G secreted in a protected microenvironment.[19,20]

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[19] 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[11] 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 Ca^{2+} 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.[36-38] 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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REFERENCES

1. Ernst E, Hammerschmidt DE, Bagge U, Matrai A, Dormandy JA: Leukocytes and the risk of ischemic diseases. JAMA 257:2318, 1987

2. Romson JL, Hook BG, Kunkel SL: Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. Circulation 67:1016, 1983

3. Mehta J, Dinerman J, Mehta P, Saldeen TGP, Lawson D, Donnelly WH, Wallin R: Neutrophil function in ischemic heart disease. Circulation 79:549, 1989

4. Henson PM: Mechanisms of release of constituents from rabbit platelets by antigen-antibody complexes and complement. II. Interaction of platelets with neutrophils. J Immunol 105:490, 1970

5. Coëffier E, Joseph D, Prévost MC, Vargaftig BB: Platelet-leukocyte interaction: Activation of rabbit platelets by FMLP-stimulated neutrophils. Br J Pharmacol 92:393, 1987

6. Oda M, Satouchi K, Yasunaga K, Saito K: Polymorphonuclear leukocyte-platelet interactions: Acetylglyceryl ether phosphocholine-induced platelet activation under stimulation with chemotactic peptide. J Biochem 100:1117, 1986

7. Virella G, Espinoza A, Patrick H, Colwell JA: Polymorphonuclear leukocytes release a factor(s) that induces platelet aggregation and ATP release after interaction with insoluble and surface-fixed immune complexes. Clin Exp Immunol 49:684, 1982

8. Chignard M, Selak MA, Smith JB: Direct evidence for the existence of a neutrophil-derived platelet activator (neutrophilin). Proc Natl Acad Sci USA 83:8609, 1986

9. Levine PH, Weinger RS, Simon J, Scoon KL, Krinsky NI:

Leukocyte-platelet interaction. Release of hydrogen peroxide by granulocytes as a modulator of platelet reactions. J Clin Invest 57:955, 1976

10. Handin RI, Karabin R, Boxer GJ: Enhancement of platelet function by superoxide anion. J Clin Invest 59:959, 1977

11. Clark RA, Klebanoff SJ: Neutrophil-platelet interaction mediated by myeloperoxidase and hydrogen peroxide. J Immunol 124:399, 1980

12. Selak MA, Chignard M, Smith JB: Cathepsin G is a strong platelet agonist released by neutrophils. Biochem J 251:293, 1988

13. Kornecki E, Ehrlich YH, Egbring R, Gramse M, Seitz R, Eckardt A, Lukasiewicz H, Niewiarowski S: Granulocyte-platelet interactions and platelet fibrinogen receptor exposure. Am J Physiol 255:H651, 1988

14. Del Maschio A, Evangelista V, Rajtar G, Chen ZM, Cerletti C, de Gaetano G: Platelet activation by polymorphonuclear leukocytes exposed to chemotactic agents. Am J Physiol 258:H870, 1990

15. Bykowska K, Kaczanowska J, Karpowicz M, Stachurska J, Kopec M: Effects of neutral proteases from blood leukocytes on human platelets. Thromb Haemost 50:768, 1983

16. Johnson KJ, Varani J: Substrate hydrolysis by immune complex-activated neutrophils: Effect of physical presentation of complexes and protease inhibitors. J Immunol 127:1875, 1981

17. Campbell EJ, Senior RM, Mc Donald JA, Cox DL: Proteolysis by neutrophils. Relative importance of cell-substrate contact and oxidative inactivation of proteinase inhibitors in vitro. J Clin Invest 70:845, 1982

18. Weiss SJ, Curnutte JT, Regiani S: Neutrophil-mediated solubilization of the subendothelial matrix: Oxidative and non oxidative mechanisms of proteolysis used by normal and chronic granulomatous disease phagocytes. J Immunol 136:636, 1986

19. Weitz JI, Huang AJ, Landman SL, Nicholson SC, Silverstein SC: Elastase-mediated fibrinogenolysis by chemoattractant-stimulated neutrophils occurs in the presence of physiologic concentrations of antiproteinases. J Exp Med 166:1836, 1987

20. Campbell EJ, Campbell MA: Pericellular proteolysis by neutrophils in the presence of proteinase inhibitors: Effects of substrate opsonization. J Cell Biol 106:667, 1988

21. Travis J, Salvesen GS: Human plasma proteinase inhibitors. Annu Rev Biochem 52:655, 1983

22. Boyum A: A one stage procedure for isolation of granulocytes and lymphocytes from human blood. General sedimentation properties of white blood cells in a 1 g gravity field. Scand J Clin Lab Invest Suppl 97:51, 1968

23. Yamaguchi A, Suzuki H, Tanoue K, Yamazaki H: Simple method of aequorin loading into platelets using dimethyl sulfoxide. Thromb Res 44:165, 1986

24. Nakajima K, Powers JC, Ashe BM, Zimmerman M: Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase. Studies with peptide substrates related to the α1-protease inhibitor reactive site. J Biol Chem 254:4027, 1979

25. Del Maschio A, Corvazier E, Maillet F, Kazatchkine MD, Maclouf J: Platelet-dependent induction and amplification of polymorphonuclear leucocyte lysosomal enzyme release. Br J Haematol 72:329, 1989

26. Lorico A, Masturzo P, Villa S, Salmona M, Semeraro N, de Gaetano G: Gentisic acid: An aspirin metabolite with multiple effects on human blood polymorphonuclear leukocytes. Biochem Pharmacol 35:2443, 1986

27. Johnson PC, Ware JA, Clieveden PB, Smith M, Dvorak AM, Salzman EW: Measurement of ionized calcium in blood platelets with the photoprotein aequorin. Comparison with quin 2. J Biol Chem 260:2069, 1986

28. Ware AJ, Smith M, Fossel ET, Salzman EW: Cytoplasmic Mg^{2+} concentration in platelets: Implications for determination of Ca^{2+} with aequorin. Am J Physiol 255:H855, 1988

29. Cerletti C, Gambino MC, Garattini S, de Gaetano G: Biochemical selectivity of oral versus intravenous aspirin in rats. Inhibition by oral aspirin of cyclooxygenase activity in platelets and presystemic but not systemic vessels. J Clin Invest 78:323, 1986

30. Escolar G, Leistikow E, White JG: The fate of the open canalicular system in surface and suspension-activated platelets. Blood 74:1983, 1989

31. Selak MA, Smith JB: Cathepsin G binding to human platelets. Evidence for a specific receptor. Biochem J 266:55, 1990

32. Seemüller U, Meier M, Ohlsson K, Muller HP, Fritz H: Isolation and characterization of a low molecular weight inhibitor (of chymotrypsin and human granulocytic elastase and cathepsin G) from leeches. Hoppe-Seyler's Z Physiol Chem 358:1105, 1977

33. Renesto P, Ferrer-Lopez P, Chignard M: Interference of recombinant eglin C, a proteinase inhibitor extracted from leeches, with neutrophil-mediated platelet activation. Lab Invest 62:409, 1990

34. Carp H, Janoff A: In vitro suppression of serum elastase-inhibitory capacity by reactive oxygen species generated by phagocytosing polymorphonuclear leukocytes. J Clin Invest 63:793, 1979

35. Weiss SJ: Tissue destruction by neutrophils. N Engl J Med 320:365, 1989

36. Jungi TW, Spycher MO, Nydegger UE, Barandun S: Platelet-leukocyte interaction: Selective binding of thrombin-stimulated platelets to human monocytes, polymorphonuclear leukocytes, and related cell lines. Blood 67:629, 1986

37. Larsen E, Celi A, Gilbert GE, Furie BC, Erban JK, Bonfanti R, Wagner DD, Furie B: PADGEM protein: A receptor that mediates the interaction of activated platelets with neutrophils and monocytes. Cell 59:305, 1989

38. Hamburger SA, McEver RP: GMP-140 mediates adhesion of stimulated platelets to neutrophils. Blood 75:550, 1990