Release of Oxygen Metabolites From Chemotactant-Stimulated Neutrophils Is Inhibited by Resting Platelets: Role of Extracellular Adenosine and Actin Polymerization

By Torbjörn Bengtsson, Stefan Zalavary, Olle Stendahl, and Magnus Grenegård

The effect of human platelets on chemotactant-induced generation of oxygen metabolites in neutrophils was investigated, using luminol-enhanced chemiluminescence (CL). Resting platelets inhibited the extracellular, but not the intracellular, production of oxygen radicals in formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe)-stimulated neutrophils. Maximal effect was obtained at the physiological neutrophil/platelet ratio of 1/50. Similar results were obtained by adding supernatants of platelets, indicating a role for extracellular adenosine deaminase (ADA), or blocking of adenosine receptors by theophylline, antagonizing the inhibitory effects of platelets on the neutrophil respiratory burst. In contrast, accumulation of adenosine by a pyrophosphorylase enhanced the inhibition. Exogenous adenosine mimicked the effects of platelets on the fMet-Leu-Phe-induced respiratory burst. To further assess the role of platelet-derived adenosine, the platelets were fixed with paraformaldehyde and incubated with the fMet-Leu-Phe-stimulated neutrophils. The inhibition was also reversed by ADA and theophylline, respectively. A prior removal of adenosine in the platelet suspension by ADA, followed by treatment with erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) to inactivate ADA, did not reverse the inhibitory action of platelets on the fMet-Leu-Phe-induced CL-response in neutrophils. However, if adenosine receptors of the neutrophil at the same time were blocked with theophylline, the inhibition was significantly reduced. Platelets markedly increased the generation of adenosine in a neutrophil suspension. This effect was antagonized by N-(4-Nitrobenzyl)-6-thioxoguanosine (NBGT), but not by 3H-adenosine monophosphate (AMP-CP), indicating that the platelet-dependent accumulation of adenosine is due to an increased release of endogenous adenosine from neutrophils and not to a degradation of extracellular AMP. In correlation, NBGT, but not AMP-CP, reversed the platelet-mediated inhibition of the fMet-Leu-Phe-induced CL-response in neutrophils. Consequently, these data suggest that a platelet-derived factor increases the release of endogenously formed adenosine from neutrophils, terminating the production of oxygen radicals. The inhibition of oxidase activity was also associated with a platelet-induced polymerization of actin in the margin of the neutrophils. Treatment of neutrophils with cytochalasin B reversed the effects of platelets, both on F-actin content and CL-response. In summary, resting platelets limit the release of oxygen radicals from chemotactant-stimulated neutrophils, thus preventing excessive damage to host tissues in the vascular space. This effect is suggested to be associated with an increased generation of neutrophil-derived adenosine enhancing an autoregulatory inhibitory pathway, and a peripheral accumulation of actin filaments forming a barrier for extracellular release of reactive oxygen radicals.

© 1996 by The American Society of Hematology.

NEUTROPHIL GRANULOCYTES play a central role in human host defense against microbial invasion. Release of reactive oxygen metabolites and lysosomal enzymes by activated neutrophils may, however, cause considerable tissue damage following ischemia and during inflammatory processes. It is, therefore, likely that there are regulatory mechanisms operating to control these destructive activities. Several endogenous, soluble inhibitors of the neutrophil respiratory burst have been described, eg, adenosine, nitric oxide, fibronectin, platelet-derived growth factor, and soluble P-selectin. Furthermore, neutrophil activation may be modulated through interaction with other cells at inflammatory sites, eg, erythrocytes, platelets, and endothelial cells.

Platelets or platelet-derived products have been shown to modify different neutrophil functions including adhesion, aggregation, chemotaxis, superoxide anion production, and degranulation. The intercellular mechanisms during platelet-neutrophil interaction are poorly characterized, but involve both soluble mediators, as well as contact-dependent mechanisms. We have recently demonstrated that coincubation of neutrophils and platelets, isolated separately from human blood, results in a cytoskeletal activation shown by sustained elevation of filamentous actin (F-actin) content in both cells. We also found that platelets markedly potentiate formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe)-induced actin assembly and calcium mobilization in neutrophils. Several studies have shown that the function of the respiratory burst oxidase, especially the extracellular release of oxygen species, as well as the secretion of granule products, are inhibited by a peripheral accumulation of actin filaments. Consequently, a platelet-mediated elevation of the neutrophil F-actin content might also affect the microbicidal systems of the neutrophil.

There is increasing evidence suggesting that adenosine nucleotides and adenosine serve as important extracellular factors modifying the biologic responses of inflammatory cells. For instance, occupation of adenosine triphosphate (ATP) receptors on neutrophils triggers mobilization of intracellular free calcium, which is considered to prime the cells for enhanced superoxide anion (O2-) responses to fMet-Leu-Phe.
Phe. Studies by Cronstein and others indicate that adenosine suppresses the neutrophil respiratory burst induced by chemoattractants through activation of a specific receptor of the A2 type. Platelets release from their dense granules substantial amounts of ATP and adenosine diphosphate (ADP), which are subsequently converted to adenosine, and may thus function as potent regulators of the oxygen radical production by activated neutrophils. In addition, adenosine derived from stimulated neutrophils is considered to function as an autocrine regulator attenuating cell activation.

In the present study, the role of platelets in the control of the neutrophil respiratory burst was investigated and related to accumulation of extracellular adenosine and activation of the actin cytoskeleton.

**MATERIALS AND METHODS**

**Materials.** The materials and their sources were as follows: dextran and Ficoll-Paque (Pharmacia, Uppsala, Sweden); sodium metrizoate (Nycomed Pharma, Oslo, Norway); bovidy phallacidin (Molecular Probes, Eugene, OR); 5-(4-Nitrobenzyl)-6-thioinosguanine (NBGT) (Research Biochemicals Int, Natick, MA); catalase (Boehringer Mannheim, Germany); adenosine, adenosine deaminase (ADA), adenosine 5' nucleotidase kit, apryrase, cytochlasin B (cyt B), cytochrome c, erythrosin-2-(hydroxy-3-nonyl)-adenine (EHNA), formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe), horseradish peroxidase (HRP), 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol), lycopophosphatidylcholine, α,β-methylene-adenosine 5'phosphate (AMP-CP), superoxide dismutase (SOD), theophylline and thrombin (Sigma Chemical Co, St Louis, MO). All reagents used were of an analytical grade.

**Cell preparation.** Polymorphonuclear neutrophil leukocytes (neutrophils) were isolated from heparinized human peripheral blood, essentially by the method of Böyum. In short, erythrocytes were removed by dextran-sodium metrizoate sedimentation and brief hypotonic lysis in distilled water. The preparation was then centrifuged on a Ficoll-paque gradient to exclude lymphocytes, monocytes and platelets. A calcium-free Krebs-Ringer phosphate buffer, supplemented with 10 mmol/L glucose and 1.5 mmol/L MgSO4 (KRG; pH 7.3), was used for washing. After isolation, the neutrophils were suspended in KRG supplemented with 1.1 mmol/L CaCl2, and stored on ice before use. Contamination of the neutrophil preparation was usually lower than 1 platelet to 10 neutrophils. Washed platelets were obtained essentially as previously described. In short, a mixture of five parts of the blood and one part of an acid-citrate-dextrose (ACD) solution was centrifuged twice for 20 minutes (2200 g followed by 480 g) and as much as possible of the plasma was removed. Finally, the platelets were resuspended in KRG and stored in plastic tubes at 4°C for 24 hours. After isolation, the platelets were stored in plastic tubes at 4°C and stained for F-actin by incubation in a mixture of bodipy phallacidin (4 U) and 2-(4-Nitrobenzyl)-6-thioguanosine (NBGT) (Research Biochemicals Int, Natick, MA); catalase (Boehringer Mannheim, Germany); adenosine, adenosine deaminase (ADA), adenosine 5' nucleotidase kit, apryrase, cytochlasin B (cyt B), cytochrome c, erythrosin-2-(hydroxy-3-nonyl)-adenine (EHNA), formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe), horseradish peroxidase (HRP), 5-amino-2,3-dihydro-1,4-phtalazinedione (luminol), lycopophosphatidylcholine, α,β-methylene-adenosine 5'phosphate (AMP-CP), superoxide dismutase (SOD), theophylline and thrombin (Sigma Chemical Co, St Louis, MO). All reagents used were of an analytical grade.

**Generation of extracellular adenosine.** The formation of extracellular adenosine in a neutrophil suspension was determined by using an enzymatic, spectrophotometrical method. The method is based on the following reactions: Adenosine deaminase (ADA) deaminates adenosine, producing inosine and ammonium ions (NH4). In a coupled reaction, catalyzed by L-glutamate dehydrogenase (GDH), NH4 reacts with 2-oxo-glutarate in the presence of reduced nicotinamide adenine dinucleotide (NADH) to form glutamate and NAD. Formation of NAD, which decreases the absorbance at 340 nm, is directly proportional to the extracellular release of adenosine. Neutrophils and platelets were incubated and mixed as described in the chemiluminescence method. The cell suspensions were then rapidly converted to cuvettes containing an assay mixture with the following composition: ADA (400 U/L), 2-oxoglutarate (3.7 mmol/L), NADH (0.2 mmol/L), GLDH (11,000 U/L), β-glycerophosphate (0.2 mmol/L), and AMP (3.2 mmol/L). The neutrophils were unstimulated or stimulated with fMet-Leu-Phe (10−7 mol/L), and the change in absorbance at 340 nm accompanying NAD formation was monitored at 37°C with a 6-channel Beckman DU-68 spectrophotometer (Beckman Instruments, Inc, Palo Alto, CA) equipped with a thermostated (37°C) cuvette holder. Scavenging of O2 by platelets or platelet products was evaluated by using the cell-free O2−-generating system of xanthine oxidase (0.02 U/mL) and hypoxanthine (0.6 mmol/L).

**Chemiluminescence.** Neutrophils and platelets were preincubated separately in multiwell-plates (Nunc Intermed, Roskilde, Denmark) at 37°C under stirring conditions. After 5 minutes, the cells were mixed (giving a final concentration of 2 × 106/mL and 2 × 109 to 1 × 1010/mL of neutrophils and platelets, respectively, and a neutrophil/platelet ratio of 1/1 to 1/50), incubated for another 2 minutes, and then stimulated with fMet-Leu-Phe (10−7 mol/L). Unstimulated cell suspensions were run in parallel. The cell suspensions were then rapidly converted to polycarbonate tubes with luminol (final concentration 50 µmol/L), stimulated with fMet-Leu-Phe and monitored for chemiluminescence (CL) at 37°C in a 6-channel Biolumat LB 9505 (Berthold Co, Wildbaden, Germany). Because peroxidase is often a limiting factor in luminol-dependent CL, extra peroxidase (HRP, 4 U) was added to the tubes in some experiments. Measurements in the HRP system reflected the total CL activity. The intracellular part of the CL response was defined as the activity obtained in the presence of SOD (200 U) and catalase (2,000 U), large molecular O2 and H2O2 scavengers, respectively. Dimethyl sulfoxide, used as a solvent vehicle for some substances, was tested and had, without exception, no effect on CL. Furthermore, inactivation of enzymes (ADA or apyrase) by boiling, totally abrogated their effects on fMet-Leu-Phe-induced CL.

**Production of superoxide anions.** O2− production was assayed spectrophotometrically by the reduction of ferricytochrome c (cyt c), essentially as described by Cohen and Chovanec. Neutrophils and platelets were incubated and mixed as described in the chemiluminescence method. The assay mixture contained 1 mL cell suspension (2 × 106 neutrophils/mL with or without 2 × 109 to 1 × 1010 platelets/mL) with 15 mg cyt c. The absorbance change accompanying cyt c reduction was monitored at 550 nm using a Beckman DU-68 spectrophotometer (Beckman Instruments, Inc, Palo Alto, CA) equipped with a thermostated (37°C) cuvette holder. Scavenging of O2− by platelets or platelet products was evaluated by using the cell-free O2−-generating system of xanthine oxidase (0.02 U/mL) and hypoxanthine (0.6 mmol/L).

**Determination of F-actin content.** Neutrophils and platelets were incubated and mixed essentially as described in the chemiluminescence assay. After various periods of time, aliquots of cell suspensions were removed, fixed with ice-cold paraformaldehyde (4%), and stained for F-actin by incubation in a mixture of bodipy phallacidin (0.6 µg/mL) and lysophosphatidylcholine (100 µg/mL). The bodipy fluorescence of individual neutrophils was determined with a cytofluorometer based on a Leitz MPV II microscope photometer.

From www.bloodjournal.org by guest on October 31, 2017. For personal use only.
PLATELETS INHIBIT NEUTROPHIL OXIDASE ACTIVITY

connected to a computer system. Only a single cell was exposed shortly (<0.8 s) at a time to avoid fading. For each sample, a minimum of 50 cells was analyzed. Background fluorescence and the fluorescence of unstained neutrophils were routinely analyzed and automatically subtracted from the fluorescence values of the cells. In comparison, we found a good correlation between the results obtained with the procedure described above and the original procedure using NBD phallacidin, respectively.

RESULTS

Platelets inhibit the extracellular release of oxygen radicals from fMet-Leu-Phe-stimulated neutrophils. The respiratory burst of neutrophil granulocytes is initiated by activation of a membrane-bound enzyme complex (NADPH-oxidase) that catalyzes the reduction of oxygen by an electron transfer from cytoplasmic NADPH. In this study, we have used luminol-enhanced chemiluminescence (CL) as an indicator for oxidase activity. Exposure of human neutrophils to fMet-Leu-Phe (10^{-7} mol/L) led to a rapid biphasic CL response (Fig 1A). The first phase (within 3 minutes) is considered to mainly reflect extracellular release of oxygen metabolites, whereas the second phase (around 5 minutes) is due to intracellular generation. Platelets alone did not show any detectable CL response on fMet-Leu-Phe stimulation, nor did they induce production of oxygen radicals by coincubated neutrophils. However, platelets dose-dependently inhibited the fMet-Leu-Phe-induced activation of the neutrophil oxidase (Fig 1A). As previously shown, regarding F-actin and [Ca^{2+}], this effect was most prominent at a physiological neutrophil/platelet (N/P) ratio between 1/50 to 1/100. At low N/P ratios (1/1 to 1/5), platelets inhibited the initial peak of the bimodal response, whereas higher concentrations of platelets affected the overall CL response (Fig 1A). Stimulating the platelets, at different N/P ratios, with thrombin (0.5 U/mL) did not significantly change the extent of inhibition of the neutrophil oxidase activity. Previous studies have shown that neutrophil chemiluminescence is dependent on cell concentration. Consequently, the observed effects by adding platelets to a neutrophil suspension might be nonspecific due to an increased cell number. However, increasing the amount of neutrophils, in cell volume equivalent with 10^9 platelets, did not mimic the inhibitory effects of platelets, but rather increased the CL-response (data not shown).

In addition to the amount of reactive oxygen species (O_2^- and H_2O_2), the luminol-amplified CL reaction depends on myeloperoxidase released from primary granules. Previous studies have indicated that the release of myeloperoxidase could be a limiting factor in the fMet-Leu-Phe-induced

---

Fig 1. Time traces of chemiluminescence emitted from fMet-Leu-Phe-stimulated neutrophils in the absence or presence of platelets. (A) Neutrophils and platelets were preincubated separately at 37°C under stirring conditions. After 5 minutes, the neutrophils (2 × 10^6/mL, final concentration) were mixed with platelets (2 × 10^6/mL, final concentration; N/P ratio 1/1 to 1/50) or with buffer (control; broken line) and incubated for another 2 minutes. The cell suspensions were then monitored for luminol-amplified chemiluminescence triggered by fMet-Leu-Phe (10^{-7} mol/L). (B) Same as (A), but in the presence of extra peroxidase, HRP (4 U). (C) Same as (A), but in the presence of the scavengers SOD (200 U) and catalase (2,000 U). Ordinate, chemiluminescence in cpm × 10^6. The figure shows representative recordings of at least five different experiments.
with buffer (control) and incubated for another 2 minutes. The cell suspensions were then monitored for luminol-amplified chemiluminescence triggered by fMet-Leu-Phe (10⁻⁷ mol/L) in the presence of extra peroxidase, HRP (4 U) (total activity) or the scavengers SOD (200 U) and catalase (2,000 U) (intracellular activity). The data are based on integral values over 10 minutes and are expressed as percent of the neutrophil control stimulated in the presence of HRP (total control). The values represent the mean ± SEM of six separate experiments.

CL response in neutrophils. It is possible that the platelet-mediated inhibition of the fMet-Leu-Phe–triggered CL response is due to a depressed granular release of myeloperoxidase. This is not likely, however, as the difference in CL response between neutrophils incubated with or without platelets remained, or was even amplified, when extra peroxidase (HRP, 4 U) was added to the system (Fig 1B). High concentrations of platelets almost totally abolished the initial peak, thus indicating that they negatively modulate the extracellular release of oxygen metabolites.

To separate the intracellular activity of the CL-response, we have used SOD and catalase. These proteins are large, membrane-impermeable O₂ and H₂O₂ scavengers, respectively. fMet-Leu-Phe stimulation of neutrophils in the presence of SOD (200 U) and catalase (2,000 U) resulted in a delayed response, without the initial peak (Fig 1C). Platelets reduced only slightly, at a 1/50 N/P ratio, the fMet-Leu-Phe–triggered CL response under these conditions (Fig 1C), i.e., platelets do not mainly interfere with the intracellular generation of oxygen metabolites. This is also illustrated in Fig 2 showing the relation between the intra and extracellular parts of the fMet-Leu-Phe–induced CL responses in neutrophils incubated in the presence of increasing numbers of platelets (analyzed on the integral values over the measuring time of 10 minutes). Whereas the extracellular release of oxygen metabolites was markedly reduced, the intracellular part of the fMet-Leu-Phe–triggered CL response was only slightly affected and proportionally increased from 18% in the control (without platelets) to about 33% in neutrophils coincubated with platelets at a 1/50 N/P ratio.

To study if similar patterns of inhibition by platelets were obtained when using another method of assaying the oxidative burst, the effects of platelets on cytochrome c reduction in stimulated neutrophils were analyzed. This method specifically monitored the extracellular release of O₂. Indeed, we found that platelets dose-dependently reduced the fMet-Leu-Phe–induced O₂ production, with an apparent inhibition at a N/P ratio of 1/5 and reaching a maximal inhibition at 1/50 (85.2% ± 14.9% and 37.2% ± 8.4%, respectively, of the control, mean ± SEM, n = 5).

**Adenosine as an extracellular messenger in the platelet-induced inhibition.** Adenosine is a powerful inhibitor of the respiratory burst of neutrophils and might be derived from the metabolism of platelet adenine nucleotides by ectonucleotidases in a mixed cell suspension. We found, by using an ATP-bioluminescent assay, that addition of resting platelets caused a threefold to fourfold increase in the extracellular concentration of ATP (from about 30 nmol/L in the control to over 100 nmol/L in the presence of platelets). fMet-Leu-Phe stimulation did not further increase the ATP level in the mixed suspension. Addition of thrombin-stimulated platelets to the neutrophil suspension raised the ATP concentration to approximately 700 nmol/L, thus showing that the platelets are potent reservoirs of adenine nucleotides. To elucidate the role of adenosine in our experiments, we pretreated the cell suspensions with either ADA, which hydrolyzes adenosine to inosine, or with apyrase, which converts ATP and ADP to AMP, presumably leading to an accumulation of adenosine. The presence of ADA in the neutrophil control increased the fMet-Leu-Phe–induced CL response with 31%, whereas apyrase had no significant effect (Fig 3). A combination of ADA and apyrase caused an enhancement of 43% (Fig 3). Addition of ADA to the platelet suspension before addition of neutrophils, or during the neutrophil-platelet incubation, markedly neutralized the suppressive effects of platelets on the fMet-Leu-Phe–induced CL response in neutrophils (Fig 3). The fMet-Leu-Phe–induced generation of oxygen radicals during a 10-minute period was instead higher in a N/P (1/50) mixture containing ADA compared with the neutrophil control (130.6% ± 7.4% of the control; mean ± SEM, n = 12). However, the time courses of the CL responses were quite different, with a delayed but markedly amplified unimodal response in the ADA-treated N/P-suspension (Fig 3). These counteracting effects of ADA were due to an increased intracellular generation of oxygen species (data not shown). This was also reflected by the observation that ADA only partly restored O₂ production measured with the cytochrome c reduction assay, which primarily registers the extracellular release of O₂ (data not shown). In contrast, apyrase exerted an additive effect on the inhibited fMet-Leu-Phe–induced CL response in neutrophils coincubated with platelets, further supporting a role for adenosine (Fig 3). The observed effects of ADA and apyrase, respectively, were not due to the solvent vehicle, as it had no effect by itself. Furthermore, the specific action of ADA was confirmed by preincubating the cells with the ADA inhibitor EHNA. This substance effectively...
blunted the effects of ADA on the fMet-Leu-Phe–induced CL response in neutrophils, both in the absence and presence of platelets (Fig 3). In addition, EHNA decreased the CL response in the neutrophil control, indicating an endogenous ADA activity (Fig 3).

To further elucidate the role of adenosine, we preincubated the neutrophils with the adenosine-antagonist theophylline. Similar to ADA, theophylline abolished the inhibitory effects of platelets on the fMet-Leu-Phe–triggered CL response in neutrophils (Fig 3). Theophylline also increased the oxygen radical production in the neutrophil control, suggesting an accumulation of adenosine in the neutrophil suspension on activation (Fig 3).

A platelet-mediated reduction of the neutrophil oxidase activity due to adenosine does not appear to involve conversion of adenosine to inosine because this nucleoside had no effect on the fMet-Leu-Phe–induced CL response in neutrophils (100.9% ± 4.6% of control, mean ± SEM, n = 3). This also argues against a role for inosine in the reversing effects of ADA on the fMet-Leu-Phe–induced CL response.

To further elucidate the role of adenosine, we preincubated the neutrophils with the adenosine-antagonist theophylline. Similar to ADA, theophylline abolished the inhibitory effects of platelets on the fMet-Leu-Phe–triggered CL response in neutrophils (Fig 3). Theophylline also increased the oxygen radical production in the neutrophil control, suggesting an accumulation of adenosine in the neutrophil suspension on activation (Fig 3).

A platelet-mediated reduction of the neutrophil oxidase activity due to adenosine does not appear to involve conversion of adenosine to inosine because this nucleoside had no effect on the fMet-Leu-Phe–induced CL response in neutrophils (100.9% ± 4.6% of control, mean ± SEM, n = 3). This also argues against a role for inosine in the reversing effects of ADA on the fMet-Leu-Phe–induced CL response.

The effects of supernatant fluids of platelets on oxidase activity in neutrophils. A role for adenosine was supported by the finding that the supernatant of the platelet suspension (1 × 10^7/mL, 9,000g for 5 minutes), devoid of intact cells, inhibited the fMet-Leu-Phe–induced CL response to the same extent (33.2% ± 3.4% of the control, mean ± SEM, n = 6) as intact platelets, and that this effect was antagonized by ADA and theophylline, respectively (105.9% ± 12.1% and 64.6% ± 1.4%, respectively, of the control; mean ± SEM, n = 6 and 3, respectively) (Fig 4). Apyrase further magnified the inhibition by the supernatant fluid (not shown). An extensive inhibition (43.8% ± 5.6% of the control, mean ± SEM, n = 4) was also achieved when mixing neutrophils with the supernatant obtained after an ultracentrifugation of the platelet suspension (100,000g, 30 minutes), an effect that was antagonized by ADA and theophylline, respectively (data not shown). However, preliminary experiments show that the inhibitory capacity of this supernatant on neutrophil oxidase activity is markedly reduced (from 55% to 20% inhibition) after passing through an ultrafilter with a molecular weight cut-off of 10 kD.

Exogenous adenosine mimics the effects of platelets. The data so far implicates adenosine as the responsible factor

---

Fig 3. The role of extracellular adenosine in the platelet-mediated inhibition of the respiratory burst in neutrophils. Neutrophils and platelets were preincubated separately at 37°C under stirring conditions. After 5 minutes, the neutrophils (2 × 10^7/mL, final concentration) were mixed with platelets (1 × 10^7/mL, final concentration, N/P ratio 1/50; P, solid lines) or with buffer (broken lines) in the absence of presence of adenosine deaminase (0.25 U/mL; ADA), apyrase (10 μg/mL; Ap), theophylline (10 μmol/L; Theo) and/or erythro-9-(2-hydroxy-3-nonyl)-adenine (10 μmol/L; EHNA) and then incubated for another 2 minutes. The suspensions were thereafter monitored for luminol-amplified chemiluminesence during stimulation with fMet-Leu-Phe (10^{-5} mol/L). The data are presented as time traces (A and B; ordinate, chemiluminesence in cpm × 10^6) or based on integral values over 10 minutes and expressed as percent of the untreated neutrophil control (C). (A) and (B) show representative recordings, and (C) the mean ± SEM of six separate experiments.
enhanced on addition of 1 μmol/L adenosine (13.8% ± 2.1% of control, mean ± SEM, n = 4), an effect that exceeded the inhibition caused by 10 μmol/L adenosine in the absence of platelets (21.0% ± 1.9% of control, mean ± SEM, n = 4). This suggests that an additional factor(s) is involved in the mechanisms behind platelet-induced restriction of neutrophil oxidase.

The effects of fixed platelets on oxidase activity in neutrophils. To further clarify the role of platelet-derived adenosine in the reduction of oxidase activity in neutrophils, we used paraformaldehyde-fixed platelets, ie, cells unable to generate adenosine. Interestingly, we found that fixed platelets, dose-dependently and to the same extent as viable platelets, inhibited the fMet-Leu-Phe–induced CL response (Fig 6). Surprisingly, the same effect was found using a supernatant of the paraformaldehyde-fixed platelets (1 × 10⁷/mL, 9,000g, 5 minutes) (Fig 7). The inhibitory effects of fixed platelets (or the equivalent supernatant fluids) were antagonized by both ADA and theophylline (Fig 7). Consequently, these experiments suggest that the platelet-mediated inhibition of the neutrophil respiratory burst is due to an increased generation of adenosine from neutrophils.

The role of neutrophil-derived adenosine in the regulation of oxygen radical production. As previously shown, activated neutrophils release adenosine, which is considered to negatively modulate cellular functions. In support, we found in this study that removal of extracellular adenosine with ADA, or blocking of adenosine receptors with theophylline, in platelet-mediated inhibition of neutrophil oxygen radical generation. In support, addition of exogenous adenosine (10⁻⁹ to 10⁻⁵ mol/L) mimicked the inhibitory effects of platelets on fMet-Leu-Phe–induced generation of oxygen radicals in neutrophils (Fig 5). The inhibition at 0.1 μmol/L of adenosine (33.2% ± 3.9% of control, mean ± SEM, n = 4) corresponded with the inhibition obtained at a N/P ratio of 1/50. Interestingly, the platelet-mediated inhibition was markedly
Platelets Increase the Generation of Adenosine from Neutrophils. The extracellular release of adenosine was analyzed with an enzymatic, spectrophotometrical assay (Fig 8). We found that a suspension of neutrophils (2 x 10^6/mL), incubated at 37°C under stirring conditions, generated adenosine at a rate of 0.17 ± 0.03 μmol/minute (mean ± SEM, n = 5). This generation was almost doubled on addition of 10^{-7} mol/L fMet-Leu-Phe (0.31 ± 0.07 μmol/minute, mean ± SEM, n = 5). Platelets (1 x 10^6/mL) markedly enhanced the release of adenosine both in an unstimulated (1.50 ± 0.20 μmol/minute, mean ± SEM, n = 5) and a fMet-Leu-Phe-stimulated (1.96 ± 0.24 μmol/minute mean ± SEM, n = 5) neutrophil suspension. Similar effects were obtained when mixing neutrophils with platelet supernatants (1 x 10^6/mL, 9,000 g, 5 minutes). The generation of adenosine in a platelet suspension was approximately 0.05 μmol/minute.

To identify the mechanisms by which adenosine is generated in neutrophil suspensions, with or without platelets, the neutrophils were treated with the 5′nucleotidase inhibitor, potentiated oxygen radical production in neutrophils triggered by fMet-Leu-Phe (Fig 3). Furthermore, inhibition of endogenous ADA activity by EHNA reduced the response (Fig 3). To further assess whether the platelet-mediated inhibition of the neutrophil respiratory burst was attributable to adenosine solely derived from neutrophils, the following experiments were performed. Viable platelets or their supernatant were treated with ADA to remove adenosine. ADA was then inactivated with EHNA, on which neutrophils were added and stimulated with fMet-Leu-Phe. This treatment did not reverse the inhibitory effects of platelets on fMet-Leu-Phe-induced oxygen radical production. However, if adenosine receptors of the neutrophil at the same time were blocked with theophylline, the inhibition was significantly reduced. This suggests that a platelet-derived factor increases the generation of adenosine from neutrophils, terminating the activation of the oxidase.

Platelets increase the generation of adenosine from neutrophils. The extracellular release of adenosine was analyzed with an enzymatic, spectrophotometrical assay (Fig 8). We found that a suspension of neutrophils (2 x 10^6/mL), incubated at 37°C under stirring conditions, generated adenosine at a rate of 0.17 ± 0.03 μmol/minute (mean ± SEM, n = 5). This generation was almost doubled on addition of 10^{-7} mol/L fMet-Leu-Phe (0.31 ± 0.07 μmol/minute, mean ± SEM, n = 5). Platelets (1 x 10^6/mL) markedly enhanced the release of adenosine both in an unstimulated (1.50 ± 0.20 μmol/minute, mean ± SEM, n = 5) and a fMet-Leu-Phe-stimulated (1.96 ± 0.24 μmol/minute mean ± SEM, n = 5) neutrophil suspension. Similar effects were obtained when mixing neutrophils with platelet supernatants (1 x 10^6/mL, 9,000 g, 5 minutes). The generation of adenosine in a platelet suspension was approximately 0.05 μmol/minute.
AMP-CP or the adenosine transport inhibitor NBGT. We found that the extracellular accumulation of adenosine, both in the absence and presence of platelets, was unaffected by AMP-CP, but reduced by NBGT (data not shown). Consequently, these data suggest that adenosine accumulation in a neutrophil suspension is not due to a 5′nucleotidase-mediated degradation of AMP, but to a release of endogenously generated adenosine. This outward transport of endogenous adenosine is obviously enhanced by platelets.

To correlate the degree of adenosine generation with oxygen radical production, the inhibitors above were used in the chemiluminescence assay. We found that NBGT potentiated the fMet-Leu-Phe–induced CL response in neutrophils, while AMP-CP was ineffective, and moreover, the platelet-mediated inhibition of the response was reversed by NBGT, but not influenced by AMP-CP (Fig 9). As a comparison, exogenous AMP (10 μmol/L) reduced the chemotactic peptide-induced generation of oxygen species, both in the absence and presence of platelets (Fig 9). However, these effects were reversed by AMP-CP. Consequently, these data indicate that neutrophils express, to a certain extent, 5′nucleotidase activity. However, an adenosine accumulation due to degradation of AMP is apparently not involved in the platelet-mediated inhibition of the respiratory burst in neutrophils.

Platelets induce actin assembly in both resting and fMet-Leu-Phe–stimulated neutrophils. The major part of the neutrophil function is dependent on a dynamic reorganization of the actin cytoskeleton. As recently reported, and shown in Fig 10, the presence of platelets induces a rapid and sustained elevation of the F-actin content in unstimulated neutrophils and markedly potentiates a subsequent actin polymerization in fMet-Leu-Phe–stimulated neutrophils.

These effects are optimal at a physiological N/P ratio of 1/50. In this study, we further characterize the effects of platelets on the morphology and F-actin distribution in coincubated neutrophils. Resting neutrophils showed an uniform cytoplasmic distribution of F-actin (Fig 11A). Stimulation with fMet-Leu-Phe (10−7 mol/L) caused an extensive transient actin polymerization in the margin of the neutrophil (Fig 11B), which thereafter became more polarized with F-actin mainly localized at one end of the cell (not shown).

Platelets induced a peripheral formation of actin filaments in coincubated neutrophils (Fig 11C), and potentiated severalfold a subsequent fMet-Leu-Phe–induced actin polymerization (Fig 11D). In these neutrophils, a broad peripheral ring of F-actin was prominent for at least 10 minutes (not shown). As indicated in Fig 11D, fMet-Leu-Phe stimulation increased the binding between neutrophils and platelets. Cytochalasin B (cyt B), which totally blocked the fMet-Leu-Phe–induced changes in both neutrophil shape and F-actin content (Fig 11E), also reduced the effects of platelets on actin polymerization in resting and fMet-Leu-Phe–stimulated neutrophils (Fig 11F). Preliminary experiments indicate that platelet supernatants increase the F-actin content in both unstimulated and fMet-Leu-Phe–stimulated neutrophils.
PLATELETS INHIBIT NEUTROPHIL OXIDASE ACTIVITY

**DISCUSSION**

Several studies indicate an intimate interaction between neutrophils and platelets, modulating their inflammatory and hemostatic functions. The present work suggests that platelets act as regulators preventing inappropriate release of reactive oxygen metabolites by activated neutrophils.

We found that resting platelets inhibit fMet-Leu-Phe—induced generation of oxygen metabolites in neutrophils, measured as luminol-enhanced chemiluminescence. These effects are not due to inactivation of fMet-Leu-Phe or inhibition of its receptor, as we have previously shown that other neutrophil responses triggered by fMet-Leu-Phe, ie, actin polymerization and calcium mobilization, are potentiated. The inhibition in chemiluminescence showed a maximum effect around a N/P ratio of 1/50, which is close to the situation in blood. Stimulation of neutrophils with fMet-Leu-Phe induces both a rapid extracellular release of oxygen metabolites and a slow, more sustained intracellular generation. Previous studies have indicated that the fMet-Leu-Phe—induced CL response in neutrophils is dependent on the presence of myeloperoxidase. It is possible that the platelet-mediated inhibition is associated with a depressed release of myeloperoxidase from primary granules of the neutrophil. However, we found that the difference in CL response between neutrophils incubated with or without platelets remained when extra peroxidase was added to the system. This indicates that platelets do not affect the release of myeloperoxidase, but instead modulate the activity of the O$_2^-$-producing NADPH-oxidase in neutrophils. In support, platelets inhibited the fMet-Leu-Phe—induced extracellular release of O$_2^-$, assayed with the cytochrome c-reduction method. Addition of SOD and catalase to the CL system enabled us to determine the intracellular activity. Interestingly, platelets only slightly reduced the intracellular part of the fMet-Leu-Phe—induced respiratory burst. Consequently, platelets inhibit the neutrophil ability to release oxygen metabolites to the extracellular milieu.

Adenine nucleotides have been reported to both enhance and inhibit O$_2^-$ production in activated neutrophils. A possible explanation for these contradictory results might be differences in how the neutrophils are exposed to adenine nucleotides and the degree of extracellular conversion to adenosine by ATPases and nucleotidases. Several investigations have suggested that adenosine functions as an extracellular messenger controlling the activity of the respiratory burst oxidase and that its effects are related to ATP and ADP released from platelets. In this study, we present several data supporting a role for adenosine as the responsible factor.

---

**Figure 10. Effect of platelets on the F-actin content in resting and fMet-Leu-Phe—stimulated neutrophils.** Neutrophils and platelets were preincubated separately at 37°C under stirring conditions. After 5 minutes, the neutrophils (2 x 10$^6$/mL, final concentration) were mixed with platelets (1 x 10$^6$/mL, final concentration; N/P ratio 1/50) or with buffer and incubated for another 2 minutes followed by stimulation with fMet-Leu-Phe (10$^{-7}$ mol/L; arrow). After various periods of time, aliquots of the cell suspension were fixed in paraformaldehyde (4%) and then selectively stained for F-actin with bodipy phallacidin. The fluorescence was quantified in single neutrophils that had been incubated in the absence (open squares) or presence of platelets (closed squares). The values represent the mean ± SEM of six separate experiments (the error bars of the points at 2 minutes lie within the size of the symbols).

---

*Platelet-mediated inhibition of oxygen radical generation is coupled to elevated F-actin levels in neutrophils.* The role of the actin cytoskeleton in the platelet-mediated effects on the neutrophil oxidase activity was studied by pretreating the neutrophils with the actin-polymerizing inhibitor cyt B. Cyt B alone did not induce any CL response, but caused an amplified and prolonged generation of oxygen radicals in fMet-Leu-Phe—stimulated neutrophils (Fig 12). The suppressive effects of platelets on the fMet-Leu-Phe—induced CL response were totally abolished in neutrophils pretreated with cyt B (Fig 12). Instead, platelets potentiated the fMet-Leu-Phe—triggered generation of oxygen metabolites in cyt B-treated neutrophils, with a maximum at a N/P ratio of 1/10 (132% ± 29% of the control; mean ± SEM, n = 4, Fig 12). Similar results were obtained when replacing cyt B with cytochalasin D (not shown).

To elucidate the relationship between changes in the actin cytoskeleton and the extracellular level of adenosine in the platelet-mediated inhibition of oxygen radical production, we analyzed the effects of different concentrations of adenosine on the fMet-Leu-Phe—induced CL response in neutrophils, treated or nontreated with cyt B (Fig 5). Although adenosine was still able to inhibit fMet-Leu-Phe—triggered production of oxygen radicals in cyt B-treated neutrophils, the sensitivity for exogenous adenosine was significantly reduced. This indicates that adenosine regulates the neutrophil oxidase activity through a mechanism involving the actin filament system. Comparison of the effects of platelets and exogenous adenosine, respectively, on the fMet-Leu-Phe—induced CL response in cyt B-treated neutrophils suggests that the platelet suspension contributes with an adenosine concentration around 10$^{-6}$ mol/L. However, because adenosine at this level reduced the CL response with only 25% in neutrophils nontreated with cyt B, this indicates that some other factor, possibly increasing the F-actin content, is involved in the platelet-induced inhibition of oxygen radical production in neutrophils.
in the platelet-mediated inhibition of the fMet-Leu-Phe-induced generation of oxygen metabolites: (1) platelets enhance the generation of adenosine in a neutrophil suspension, (2) removal of adenosine by ADA reversed, whereas accumulation of adenosine by apyrase potentiated the platelet-mediated effects, (3) blocking of adenosine receptors of the neutrophil with the adenosine receptor antagonist theophylline abolished the inhibitory effects of platelets, (4) a supernatant fluid of the platelet suspension had the same effect as intact cells, an effect that was reversed by ADA and theophylline, respectively, and (5) addition of exogenous adenosine mimicked the effects of platelets. The counteracting effects of ADA on the platelet-mediated inhibition of the neutrophil oxidase activity might be due to an accumula-
PLATELETS INHIBIT NEUTROPHIL OXIDASE ACTIVITY

Fig 12. Chemiluminescence emitted from cyt B-treated neutrophils stimulated with fMet-Leu-Phe in the absence or presence of platelets. Neutrophils (2 × 10⁶/mL, final concentration), pretreated with cyt B (5 μg/mL) for 5 minutes, were incubated in the absence (control; broken line) or presence of platelets (2 × 10⁶ to 1 × 10⁷/mL, final concentration; N/P ratio 1/1 to 1/50) for 2 minutes and then monitored for luminol-amplified chemiluminescence triggered by fMet-Leu-Phe (10⁻⁷ mol/L). The data are presented as time traces (A; ordinate, chemiluminescence in cpm × 10⁴) or based on integral values over 10 minutes and expressed as percent of the neutrophil control (B). (A) shows representative recordings, and (B) the mean ± SEM of five separate experiments.

tion of inosine and hypoxanthine, ie, the products of deamination of adenosine. However, neither inosine (this study) nor hypoxanthine affect fMet-Leu-Phe–induced production of oxygen radicals in neutrophils.

To further assess the role of adenosine and other soluble factors in the platelet-mediated inhibition, the platelets were fixed with paraformaldehyde and then thoroughly washed. These cells had no metabolism and were thus incompatible to generate adenosine and its related metabolites. Interestingly, fixed platelets, as well as their equivalent supernatants, inhibited the fMet-Leu-Phe–triggered production of oxygen radi-
cals to the same extent as viable platelets and, furthermore, these effects were antagonized by ADA and theophylline, respectively. Pretreatment of a suspension of viable platelets (or the equivalent supernatant) with ADA to remove extracellular adenosine, followed by EHNA to inactivate ADA, did not reverse the inhibitory effects on the fMet-Leu-Phe–induced production of reactive oxygen species in neutrophils. However, if adenosine receptors of the neutrophil at the same time were blocked with theophylline, the inhibition was significantly reduced. Consequently, these experiments suggest that platelets induce a generation of adenosine or its related metabolites from coincubated neutrophils. It has previously been shown that neutrophils can be stimulated, eg, by fMet-Leu-Phe, to release adenosine and related nucleotide and nucleoside metabolites. In this study, we found that incubation of neutrophils at 37°C under stirring conditions is associated with a release of adenosine, which is markedly amplified in the presence of platelets. This release reaction has been suggested to be an autoregulatory loop, where adenosine bind to A₂-receptors on the neutrophil surface and thereby inhibit further neutrophil activation, presumably by elevating the intracellular cAMP-level. This is also supported in our study, where removal of extracellular adenosine (with ADA) or blocking of adenosine receptors (with theophylline) potentiate fMet-Leu-Phe–induced respiratory burst in neutrophils, whereas inhibition of endogenous ADA activity of the neutrophil (with EHNA) instead reduces the response. Furthermore, we found that the extracellular accumulation of adenosine was reduced by the adenosine-transport inhibitor NBGT, and that this effect was associated with an increased production of oxygen metabolites in fMet-Leu-Phe–stimulated neutrophils, both in the absence and presence of platelets. In contrast, the 5’nucleotidase inhibitor AMP-CP was unable to affect either adenosine generation or oxygen radical production, thus arguing against a role for adenosine formed by degradation of extracellular AMP. In conclusion, this study indicates that a platelet-derived factor increases the release of endogenously generated adenosine from neutrophils, leading to a termination of the respiratory burst.

The actin filament network may regulate the activity of the respiratory burst oxidase. We and others have shown that inhibitors of actin polymerization potentiate agonist-induced generation of oxygen radicals. Furthermore, Woodman et al. have suggested not only a functional, but even a physical link, between the respiratory burst oxidase and the actin filament system. Consequently, our observation that platelets induce a peripheral accumulation of F-actin in resting and fMet-Leu-Phe–stimulated neutrophils might further explain the inhibitory effect on fMet-Leu-Phe–induced CL in neutrophils. This idea was tested by mixing platelets with neutrophils pretreated with cyt B, followed by fMet-Leu-Phe stimulation. We found that both the formation of actin filaments and the inhibition of oxidase activity induced by platelets were abolished. The extensive peripheral accumulation of actin filaments in neutrophils incubated with platelets might thus inhibit the movement and the assembly of the different components leading to a functional oxidase, and thereafter its access to the plasma membrane for extra-
cellular release of reactive oxygen species. Recent data also show that the platelet-induced effects on the actin cytoskeleton in the neutrophil correlate with a potentiated phagocytosis of IgG-opsonized particles.\textsuperscript{40}

Several data presented in this study disagree with the idea that the inhibitory effects of platelets on oxygen radical generation in neutrophils are due to mechanisms of platelets consuming oxygen metabolites, eg, superoxide dismutase activity or the glutathione cycle: (1) platelets did not affect O$_2^-$ production in a cell-free system in which O$_2^-$ was generated with xanthine-xanthine oxidase, (2) the platelet-mediated inhibition was reversed by ADA and theophylline, respectively, and (3) no significant inhibition was observed by platelets on neutrophils pretreated with cyt B. Furthermore, we have recently shown that platelets markedly enhance the oxidative burst triggered by IgG-opsonized yeast particles in human neutrophils.\textsuperscript{40}

The intracellular processes underlying adenosine regulation of neutrophil activities are still uncertain, but have been suggested to involve changes in receptor activity, cytoskeletal dynamics, and generation of intracellular messengers.\textsuperscript{37} We have recently shown that adenosine, through A$_3$-receptor activation, inhibits FcR-mediated phagocytosis by elevating the intracellular concentration of cyclic AMP.\textsuperscript{41} However, a role for adenosine-triggered elevation of cAMP in the inhibitory effects by platelets is less possible, as cAMP has been shown to inhibit increases in both F-actin content and cytosolic free calcium in fMet-Leu-Phe–triggered neutrophils,\textsuperscript{42,43} responses shown to be potentiated in the presence of platelets.\textsuperscript{17} Cronstein et al\textsuperscript{14} have suggested that adenosine inhibits fMet-Leu-Phe–induced O$_2^-$ production by promoting a rapid association of occupied fMet-Leu-Phe receptors with the cytoskeleton. This idea is attractive as we found that (1) platelets increase the submembraneous actin cytoskeleton, which might favor an adenosine-induced receptor-cytoskeleton association, and (2) the suppressive effects by platelets, as well as by exogenous adenosine, on the fMet-Leu-Phe–induced CL response are reduced when actin polymerization in neutrophils is inhibited by cyt B. However, we have previously shown that platelets markedly potentiate other fMet-Leu-Phe–induced processes, eg, calcium mobilization,\textsuperscript{17} thus arguing against a mechanism involving an inactivation of the fMet-Leu-Phe receptor. Consequently, our study suggests that the effects of platelets are attributable to an accumulation of adenosine inhibiting the neutrophil oxidase activity through a pathway yet unidentified. The nature of the platelet-derived factor, generating adenosine from neutrophils, as well as the mechanisms of action of adenosine, require further studies.

In summary, we report that resting platelets inhibit the neutrophil ability to release reactive oxygen metabolites in response to a chemoattractant. We suggest that this effect is attributable to a platelet-derived factor, which increases the generation of adenosine from neutrophils and thereby enhances an autocrine inhibitory pathway. Furthermore, the inhibition is associated with an increased peripheral formation of actin filaments, which might form a barrier for an extracellular release of oxygen radicals. These platelet-related defense mechanisms might be relevant in controlling the harmful consequences of an excessive neutrophil activation during hemostasis and inflammation.

\textbf{REFERENCES}

18. Al-Mohanna FA, Hallet MB: Actin polymerization modifies...


Release of oxygen metabolites from chemoattractant-stimulated neutrophils is inhibited by resting platelets: role of extracellular adenosine and actin polymerization

T Bengtsson, S Zalavary, O Stendahl and M Grenegard