Regulation of polymorphonuclear leukocyte function by platelets

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ABSTRACT

Objective: To investigate the full effect of platelet-derived constituents on various polymorphonuclear neutrophil leukocyte responses.

Methods: Polymorphonuclear neutrophil leukocytes and platelets were separated from fresh blood of normal healthy volunteers. Platelets were then stimulated partially, or maximally to release constituents of their α- or α- and dense granules. The effects of these constituents on polymorphonuclear neutrophil leukocyte function (oxidase activity, degranulation and migration) were investigated.

Results: Platelet-derived constituents were found to both enhance, and inhibit polymorphonuclear neutrophil leukocytes-oxidant production, depending on the incubation time. Enhancement was due to dense granule-derived nucleotides (adenosine diphosphate and adenosine diphosphate), while inhibition was due to adenosine monophosphate derived from these nucleotides by polymorphonuclear neutrophil leukocyte surface nucleotidases. This latter inhibitory effect was reversed by the cytokine, granulocyte-macrophage colony stimulating-factor. Moreover, platelet constituents consistently enhanced other polymorphonuclear neutrophil leukocyte responses including degranulation and migration regardless of the incubation period. The latter enhancement was due to α-granule constituents, most likely platelet factor 4.

Conclusion: Platelets, through release of their granular constituents, are able to modulate polymorphonuclear neutrophil leukocyte function in a way that is physiologically beneficial.

Keywords: Inflammation, polymorphonuclear neutrophil leukocytes, platelets, granulocyte-macrophage colony stimulating-factor, nucleotides, α-granules, dense granules.

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Polymorphonuclear leukocytes (PMN neutrophils) and platelets play important roles in inflammation and hemostasis. Polymorphonuclear leukocytes are highly mobile cells and are the first to respond to invading microorganisms. These cells therefore predominate in acute inflammation and are often sufficient to control and eliminate the invading organisms. Platelets on the other hand respond instantaneously to damaged blood vessels by activating and aggregating to form hemostatic plugs that prevent blood loss. The function of each cell type has been examined in isolation, and the processes of inflammation and hemostasis have been viewed separately. However, the observations that platelets and PMN are found in close proximity in a number of inflammatory reactions including bacterial endocarditis, atherosclerosis, and the adult respiratory distress syndrome[1-3] has prompted the idea that platelets could play a role in inflammation. Direct evidence for the participation of platelets in inflammation comes from animal experiments. Thus, depletion of platelets prevents the destruction of pulmonary microvesculture following thermal injury to the skin or complement activation.[4-5] These in-vivo observations stimulated a wide interest in the study of platelet-PMN interactions in-vitro. In-vitro, platelets

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were shown to either enhance,[6-7] or inhibit[8-10] PMN function. In our previous studies we have shown that platelets enhanced n-formyl-methionyl-leucyl-phenylalanine (FMLP) - stimulated PMN oxidant production through the selective release of α–granule constituents without the significant secretion of the nucleotide-containing dense granules. Moreover, the α–granule-factor was identified as platelet factor 4 (PF4).[7] In a more recent study, we showed that maximally stimulated-platelets (made to release both α- and dense granules) had a dual effect on PMN oxidase function. Short incubation of PMN with supernatants from maximally stimulated platelets initially enhanced PMN function, but longer incubation, lead to inhibition.[11] The initial enhancement was due to dense granule-derived adenosine triphosphate (ATP) and adenosine diphosphate (ADP). The latter inhibition was due to adenosine monophosphate (AMP) produced from ATP and ADP by the action of PMN ectonucleotidases.[11] The effects of maximally stimulated platelets on other PMN responses (degranulation, migration) were not studied. In the present study, we have examined the effect of maximally stimulated platelets on other PMN responses so as to provide a more complete picture of the interaction of platelets and PMN. In the present study, we show that maximally stimulated platelets, unlike their effects on the oxidase, always enhanced other PMN responses. The relevance of these and previous results will be discussed in relation to the possible general inflammatory reaction.

Methods. ***Isolation of cells from whole blood.*** Fresh citrated normal venous blood (12.9 mM tri-sodium citrate) was centrifuged for 10 mins at 200g. After removal of platelet-rich plasma (PRP) the residue of blood was reconstituted to the original volume with 6% solution of Dextran T500 (Pharmacia LKB Biotchnology AB, Uppsala, Sweden) in phosphate-buffered saline (PBS) pH 7.3, and then left at 37°C for 30 mins to sediment red blood cells.

Isolation of PMN. The cell suspension above the sedimented red cells was collected and placed on top of a discontinuous Ficoll-Hypaque density gradient. The gradient was prepared from undiluted Mono-Poly Resolving Medium (ICN Biomedicals, Bucks.) which was diluted with 15% PBS. After centrifugation for 30 mins at 500g at room temperature, > 98% pure PMN was collected from the interface between the gradient solutions and washed once in Hanks balanced salt solution (HBSS) (Flow Laboratories, Scotland), supplemented by 12.9 mM ▪tri-sodium citrate. The cells were then resuspended first in 0.5 ml citrated HBSS, counted and diluted to a concentration of 10^7 cells/ml with RPMI medium (Northumbria Biologicals Ltd).

Isolation of platelets. To separate platelets from the plasma, PRP was centrifuged for 30 mins at 500g at room temperature on a discontinuous albumin density gradient consisting of 340 mg/ml and 250 mg/ml bovine serum albumin (BSA) (Sigma Chemical Co.) solutions in citrated HBSS. Platelets were collected from the interface between the 2 albumin solutions and washed once in citrated HBSS. The cells were gently resuspended in 0.5 ml of citrated-HBSS, counted and finally diluted to the desired concentration with RPMI medium.

Platelet stimulation. A sample containing 5 x 10^8 platelets suspended in 1 ml RPMI medium was prewarmed to 37°C in aggregometer tubes, then mixed with 0.5 or 25 ug/ml collagen (Hormon Chemie, Germany) and continuously stirred (700 rpm) for 6 mins. These 2 concentrations of collagen induced partial cell activation α-granule release) and full cell activation (release of both α- and dense granules).[11] At the end of stimulation, platelet aggregates were separated from the medium by centrifugation (1 min at 13000g in a microcentrifuge) and the supernatant used for PMN priming.

Measurement of lactate dehydrogenase. Lactate dehydrogenase (LDH) release was employed as a control of the selectivity of platelet granule secretion without the loss of cytoplasmic proteins. The enzyme activity was measured according to the instruction of the reagent supplier (Sigma). The activity of LDH in the supernatants of collagen stimulated platelets, when expressed as a percentage of total LDH activity found in the supernatant of platelets lyzed by twice freezing and thawing, was approximately1%.

PMN priming. 1 x 10^6 PMN suspended in 200 ul RPMI medium in Eppendorf tubes were primed by incubation at 37°C for 20 mins with the following: 1 x 10^7 platelets, supernatants from an equivalent number of platelets stimulated with different concentrations of collagen, 50 uM ATP, 50 uM ADP, or 50 uM AMP ± 0.5 nM granulocyte-macrophage colony stimulating-factor (GM-CSF) (Glaxo Pharmaceuticals). The latter concentration was found to produce optimal priming effects on PMN.[12]

Cytochrome C reduction assay. Oxygen production was measured by superoxide dismutase-inhibitable cytochrome C reduction.[13] The amount of reduced cytochrome C was measured spectrophotometrically at 550 nm, using an extinction coefficient of 21.1 mm l^{-1}/cm.[14]

Degranulation. Cluster of differentiation 11b (CD11b) was used as a maker for PMN degranulation.[15] Polymorphonuclear leukocytes alone or with platelets or platelet derived materials was incubated at 37°C for 20 mins before stimulation with FMLP (10^{-6} M) for another 15 mins. Following stimulation, PMN were fixed with 1% paraformaldehyde in PBS for 30 mins at room temperature to stop the reaction. Cells were then centrifuged, and supernatants decanted and cells

washed twice in fluoresciene activated cell sorter (FACS) washing buffer consisting of 1% BSA in PBS. A monoclonal anti CD11b antibody (Becton Dickinson, UK) was added and incubated for 30 mins at 4°C. Following incubation the cells were washed 3 times and incubated with GAM-FITC (Becton Dickinson) for another 30 mins. Finally, the cells were washed 3 times and analyzed by flow cytometry (Becton Dickinson FACS analyzer with Consort 30 computer program). Mean fluorescence was measured on a log scale.

Migration assay. Polymorphouclear leukocytes were suspended in RPMI medium at $2x10^6$ ml. Thirty ul of RPMI medium was pipetted into the bottom wells of a 48-well chemotaxis chamber (Neuro Probe, Bethesda, MD). The upper chamber was then placed on top, with a 5μm pore presoaked nitro-cellulose filter between the upper and the lower wells. Sixty ul of solution containing PMN ± supernatants from variously stimulated platelets, ATP, ADP or AMP was added to the upper wells and then incubated for 2 hours at 37°C in 5% carbon dioxide. After this period, the filters were removed, washed once in PBS, and then fixed in paraformaldehyde solution (1:1 37% paraformaldehyde:water). Filters were stained with hematoxylin, and cell migration was assessed using the second-cell leading-front technique on a calibrated microscope.[16]

Results. ***Effects of platelet-derived constituents on PMN oxidase.*** In our recent study we showed that supernatants from maximally stimulated platelets (S**) had a dual effect on PMN oxidase activity as measured by chemilumenicsence assay. Short incubation of PMN with S** enhanced the oxidase response while, longer incubation inhibited such response. Enhancement was attributed to platelet-derived nucleotides ATP and ADP, while subsequent inhibition was attributed to AMP generated from these nucleotides by the action of PMN ectoneucleotidases.[11] In the present study, we were able to confirm our previous findings using a different assay to monitor the oxidase activity (Cytochrome C reduction assay). Table 1 shows that S** and ATP enhanced PMN oxidase at 1 min of incubation, while AMP inhibited such a response. With longer incubation (30 min), both S** and ATP became inhibitory on the PMN oxidase and this inhibition was similar to that seen with AMP (Table 1). Adenosine diphosphate had a similar effect on PMN to that observed with ATP (results not shown). Whole platelet suspensions as well as supernatants from partially stimulated platelets (S*) had very little effect on PMN oxidase at both time points (results not shown). These, and our previous results,[11] clearly show that S** have a dual effect on PMN oxidase. The initial enhancement is due to the dense granule-derived ATP and ADP while the latter inhibition is due to AMP derived from these neucleotides by PMN ectoneucleotides. Next we investigated the effect of platelet supernatants and purified neucleotides on other PMN responses (degranulation and migration).

Table 1 - Effect of platelet-derived constituents and purified nucleotides on PMN O^2 production.

Variable	O_2^- production (nmol/10^6 PMN/min.) 1 min.	30 mins.
PMN alone	5.84 ± 0.41	5.90 ± 1.00
PMN + S**	8.20 ± 0.94	2.80 ± 0.17
PMN + ATP	7.03 ± 0.83	2.50 ± 1.5
PMN + AMP	2.50 ± 0.50	1.25 ± 1.0

O_2^-=superoxide, PMN=polymorphonuclear neutrophil leukocytes, S**=maximally stimulated platelets, ATP=adenosine triphosphate, AMP=adenosine monophosphate, mins=minute(s)

Enhancement of PMN degranulation by platelet-constituents. Stimulation of PMN with classical agonists such as FMLP leads to degranulation of PMN primary and secondary granules. CD11b, a very important PMN adhesion molecule, interacts with the intracellular adhesion molecule (ICAM) on the surface of the endothelium and mediates strong adhesion and subsequent PMN transmigration across the endothelium to sites of infection. This molecule is found stored in PMN secondary granules, and upon PMN stimulation becomes translocated to the cell surface. Therefore the expression of this molecule on PMN surface has been taken as a measure of PMN degranulation.[15,17] We therefore used CD11b expression to monitor PMN degranulation. Polymorphonuclear leukocytes were

Table 2 - Effect of platelet-derived constituents on PMN degranulation.

Variable	MFI
PMN alone	219 ± 14
PMN + PL	445 ± 19
PMN + S*	371 ± 8
PMN + S**	385 ± 12
PMN + ATP	270 ± 15
PMN + AMP	224 ± 17

PMN-polymorphonuclear leukocyte, ATP=adenosine triphosphate, AMP=adenosine monophosphate, MFI=mean fluorescence intensity, PL=whole platelet suspensions, S*=partially stimulated platelets, S**=maximally stimulated

Table 3 - Effect of platelet-derived constituents and purified nucleotides on PMN migration.

Variable	Migration (μm)
PMN alone	30 ± 1.5
PMN + PL	53 ± 2.5
PMN + S*	55 ± 1.8
PMN + S**	58 ± 2.0
PMN + ATP	32 ± 1.3
PMN + AMP	31 ± 1.9

PMN=polymorphonuclear neutrophil leukocytes, PL=whole platelet suspensions, S*=partially stimulated platelets, S**=maximally stimulated platelets, ATP= adenosine triphosphate, AMP= adenosine monophosphate

Table 4 - GM-CSF reverse the inhibitory effect of AMP on PMN oxidase activity.

Variable	O_2^- production (nmol/10^6 PMN/min)	
	- GM-CSF	+ GM-CSF
PMN	5.9 ± 1.00	16.58 ± 1.21
PMN + S**	1.3 ± 0.22	8.59 ± 1.35
PMN + AMP	2.5 ± 0.19	9.1 ± 1.10

PMN=polymorphonuclear neutrophil leukocytes, O_2^-=superoxide, nmol=nano moles, GM-CSF=granulocyte-macrophage colony stimulating-factor, S**=maximally stimulated platelets,AMP=adenosine monophosphate

incubated with platelets, or platelet-derived constituents before stimulation with FMLP and measurements of CD11b expression by flow cytometry. As can be seen from Table 2, both whole platelet suspensions (PL) as well as supernatants from platelets made to release their α-granules only, S* enhanced PMN degranulation. Supernatants from platelets made to release both of their granules had no further increase in PMN CD11b expression. Purified ATP (and ADP, results not shown) had slight enhancement, while purified AMP was without any effect. These results clearly show that firstly, platelets enhance PMN degranulation through the release of α-granule constituents, most likely PF4.[7] Secondly, unlike their effects on PMN oxidase, S** and AMP had no inhibitory effect on the PMN degranulation process.

Enhancement of PMN migration by platelet-products. In order for PMN to perform their function, they must firstly migrate from the blood vessels across the endothelium into sites of infection. The process of migration is mediated by adhesion-receptors found on the surfaces of both PMN and the endothelium. Next, we therefore investigated the effect of platelet-products on PMN migration using the Boydon chamber. As can be seen from Table 3, incubation of PMN with PL, or S* enhanced PMN migration, while S** had no further effect on PMN migration. Purified ATP (and ADP, not shown) and AMP had no effect on PMN migration (Table 3). These results again show that platelet α-granule-constituents play a major role in the enhancement of PMN-migration, while the dense granule constituents had very little effect. As with degranulation, AMP had no inhibitory effect on PMN migration.

Reversal of AMP-inhibitory effects on PMN-oxidase by GM-CSF. During inflammation, a number of cytokines are produced by hematopoeitic and non hematopoitic cells including fibroblasts, endothelium and macrophages.[18] These cytokines have a major modulatory effect on PMN responses. We, and others, have shown that one of these cytokines, GM-CSF induces marked enhancement of PMN oxidase activation. Here, we have investigated the possibility that the inhibitory effects of platelet-derived AMP on PMN oxidase activity could be reversed by GM-CSF. Indeed, as can be seen from Table 4, GM-CSF was able to reverse the inhibitory effects of both the supernatants from S** as well as purified AMP on PMN oxidase-activity.

Discussion. Platelet-neutrophil interaction is now a well accepted phenomena. Evidence for such interaction has accumulated both from in-vivo and in-vitro studies. In-vivo association of platelets and neutrophils has been observed in a variety of pathological situations.[1-3] Direct in-vivo evidence for such interaction has come from animal experiments where depletion of platelets prevents damage of pulmonary microvasculture following infusion of endotoxin or thermal injury to the skin.[4] These observations stimulated great interest in the in-vitro study of platelet-neutrophil interaction. Thus, we and others have shown that platelets could enhance neutrophil-oxidase activity in response to stimulation by FMLP[6-7] while others have shown that platelets inhibited such response.[8-10] These apparent contradictions were resolved by our recent study where we showed that platelets could both enhance and inhibit PMN oxidase function depending on the activation state of platelets. The inhibitory effect was attributed to the effect of AMP derived from platelet nucleotides.[11] In the present study, we confirm our previous findings and further show that platelet-derived α-granule and dense granule-

constituents have different effects on PMN responses. Firstly, we show that platelets are able to enhance PMN degranulation and migration through the release of α-granule-constituents, most likely PF4.[7] Secondly, dense granule constituents as well as purified neucleotides had very little effect on these 2 PMN reactions. Thirdly, AMP which inhibits PMN oxidase activity, had no inhibitory effect on these responses. Lastly, we were able to show that the inhibitory effect of AMP on PMN oxidase could be reversed by the action of cytokines such as GM-CSF. These findings have major relevance to some in-vivo inflammatory situations. Injury to blood vessels would lead to damage of the endothelium with exposure of collagen in the subendothelium, leading to platelets binding to collagen and subsequent full activation. Released platelet α-granule constituents such as PF4 would be expected to induce PMN degranulation and migration. Degranulation of PMN would lead to the expression of CD11b on their surfaces. This receptor would then cause PMN binding to ICAM-1 on the endothelium and mediate adhesion and subsequent migration across the endothelium and subendothelium matrix towards the site of infection. The fact that platelet constituents potentiate PMN degranulation and migration would be physiologically beneficial, since it would enable PMN to leave the blood vessels and get to sites of infection. On the other hand, full activation of PMN in circulation with resultant generation of oxidants in the blood vessels and during adhesion to the endothelium while still away from sites of infection would be undesirable, as it would lead to tissue damage. Therefore, inhibition of PMN oxidase by platelet derived AMP while still on route to sites of infection would also be physiologically desirable. However, once PMN have migrated across the blood vessels to sites of infection, continuous inhibition of PMN oxidase would be undesirable. Under these circumstances, cytokines such as GM-CSF released from adjacent cell such as macrophages and fibroblasts[18] would potentiate PMN oxidase and override the inhibitory effect of platelet derived AMP. In the absence of pathogens (for example sterile injuries), cytokines would not be expected to be released and PMN oxidase activity would continue to be inhibited by platelet-derived AMP. This would result in reduced tissue destruction.

Thus, platelets appear to modulate PMN responses so that PMN function is potentiated or inhibited at the right time and in the right place.

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