Human Blood Platelets, PMN Leukocytes and Their Interactions *in vitro*. Responses to Selective and Non-selective Stimuli

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Abstract. Using simultaneous recording of aggregation and chemiluminescence, responses of human polymorphonuclear leukocytes, blood platelets and their mixture were investigated after stimulation by specific as well as non-specific stimuli for each cell. In our experimental settings, aggregation of platelets and PMN leukocytes was increased in the following order of stimuli: PMA<A23187<thrombin and FMLP<A23187<PMA, respectively. FMLP selective for PMN leukocytes did not activate platelet aggregation, and, on the other hand, aggregation of PMN leukocytes was not induced by thrombin. The presence of PMN leukocytes decreased aggregatory responses of platelets to all the stimuli applied. Luminol amplified chemiluminescence of PMN leukocytes was increased in the order: thrombin<FMLP< A23187<PMA, but platelets alone did not show detectable chemiluminescence response to any stimulation. Platelets significantly decreased chemiluminescence of PMN leukocytes and their inhibitory effect did not depend on the type of stimulation. These observations suggest that under defined experimental conditions human PMN leukocytes and platelets might decrease mutually their dominant responses *in vitro*. The inhibitory effect was dependent either on the selectivity of stimuli, or on cell to cell contact before stimulus addition.

Key words: Human polymorphonuclear leukocytes — Human blood platelets — Interactions — Aggregation — Luminol-amplified chemiluminescence

Introduction

PMN leukocytes and platelets are important components of the blood, involved in haemostasis, thrombosis and inflammatory processes (Bengtsson and Grenegård 1994). Blood platelets represent the first line of host defense when normal vessels are injured. Platelet adhesion to subendothelium, aggregation and further platelet

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recruitment are involved in haemostatic plug formation. The main function of human neutrophils is to detect, approach and destroy invading microorganisms. The killing of microorganisms in phagocytic vesicles occurs by release of microbicidal compounds from various populations of granules and by generation of highly reactive oxygen metabolites. The close location of PMN leukocytes and platelets in several pathophysiological processes, such as haemostasis and inflammation, leads to cellular interactions that can modulate these events. Moreover, it has been suggested that *in vitro* platelets could change responses of neutrophils to specific and unspecific stimuli, and *vice versa* (Cerletti et al. 1992; Bazzoni et al. 1992; Del Maschio et al. 1993). In general, controversial results were reported in different experimental conditions employed. Thus, evaluation of responses of PMN leukocytes, platelets and interactions between them under defined experimental conditions may represent an important basis to study pathophysiological events and their pharmacological correction.

The luminol-amplified chemiluminescence technique is widely used to study the generation of highly reactive oxygen metabolites in PMN leukocytes (De Chatelet et al. 1982; Dahlgren et al. 1989). Aggregation of blood platelets and PMN leukocytes can be monitored by light transmission from cell suspension in a platelet aggregometer. In the present work we used lumi-aggregometer simultaneously to monitor the increase of light transmission as a consequence of aggregation and the luminol-enhanced chemiluminescence as a measure of the oxidative burst. Selective as well as non-selective stimuli were applied for each cell: FMLP (selective for PMN leukocytes), thrombin (non-selective with preferential stimulation of aggregation of platelets), A23187 and PMA (non-selective, bypassing membrane receptors). Under the same defined experimetal conditions we investigated responses of a) monocultures of cells and b) interactions between human blood platelets and PMN leukocytes in dependence on the incubation mode before addition of the corresponding stimulus.

Materials and Methods

N-formyl-methionyl-leucyl-phenyl-alanine – FMLP, phorbol 12-myristate 13-acetate – PMA, dextran, 5-amino-2,3-dihydro-1,4-phthalazinedione-luminol (Sigma-Aldrich Chemie, Germany), A23187 (Calbiochem, Switzerland), Lymphoprep (Nycomed, Pharma AS, Norway), human thrombin (Imuna, Šarišské Michaľany, Slovakia). All other chemicals of analytical grade were from available commercial sources.

Isolation

In all experiments blood platelets and PMN leukocytes were obtained from whole blood of healthy volunteers (men, aged 20 to 50 years). Blood was withdrawn into 3.8 % v/w disodium citrate dihydrate (9 : 1). PMN leukocytes were isolated by modified standard Böyum's method (Böyum 1968). Briefly, erythrocytes were allowed to sediment in dextran and PMN leukocytes were separated by gradient

centrifugation on Lymphoprep ($500 \times g$, 30 min, 22 ℃). After hypotonic lysis of contaminating erythrocytes, PMN leukocytes were washed and resuspended in calcium and magnesium free phosphate buffer saline PBS (in mmol/l: 137 NaCl; 2.7 KCl; 8.1 Na_2HPO_4; 1.5 KH_2PO_4; pH = 7.4), at a final concentration of 10^7PMN/ml. The obtained cell suspension contained more than 96 % viable cells as evaluated by trypan blue.

Platelets were isolated by differential centrifugation of platelet-rich plasma (Nosáľ et al. 1997). Sedimented platelets were resuspended in Tyrode solution (mmol/l: 137 NaCl; 2.7 KCl; 12 $NaHCO_3$; 0.4 NaH_2PO_4; 1 $MgCl_2$; 5.4 EDTA; 5.6 dextrose; pH = 6.9) to obtain 2×10^8 platelets/ml.

Aggregation and chemiluminescence

1) Isolated human platelets (10^8/500 μl Tyrode solution + 470 μl PBS with 1.8 mmol/l $CaCl_2$ and 0.5 mmol/l $MgCl_2$) or PMN leukocytes (2×10^6 PMN/470 μl PBS with 1.8 mmol/l $CaCl_2$ and 0.5 mmol/l $MgCl_2$ + 500 μl Tyrode solution) were preincubated 3 min at 37 ℃. After incubation for 3 min at 37 ℃ with 20 μl luminol (final concentration 5 μmol/l), 10 μl of the appropriate stimulus was added (under constant stirring at 1000 rpm): 0.1 μmol/l FMLP, 0.05 NIH units/ml thrombin, 0.5 μmol/l calcium ionophore A23187 or 0.01μmol/l PMA.

2) Isolated human platelets (10^8/500 μl Tyrode solution) and PMN leukocytes (2×10^6/470 μl PBS with 1.8 mmol/l $CaCl_2$ and 0.5 mmol/l $MgCl_2$) in the physiological ratio 1 : 50 were preincubated together (A) for 3 min at 37 ℃ and for further 3 min with 20 μl luminol (final concentration 5 μmol/l) (under constant stirring at 1000 rpm) before adding the stimulus, or (B) PMN leukocytes and platelets were incubated separately for 6 min, and mixed with 10 μl of appropriate stimulus: 0.1 μmol/l FMLP, 0.05 NIH units/ml thrombin, 0.5 μmol/l calcium ionophore A23187 or 0.01 μmol/l PMA.

Aggregation and luminol-enhanced chemiluminescence were recorded in the sample simultaneously in Lumi-aggregometer Chrono-Log for 6–15 min at the appropriate sensitivity setting. The chemiluminescence curve after FMLP, A23187 or PMA was evaluated as the peak of the chemiluminescence curve [mV]. After thrombin stimulation, the chemiluminescence curve failed to reach the maximum and chemiluminescence was evaluated 5 min after addition of the stimulus. Aggregation was evaluated as the amplitude of the aggregation curve at the 5^{th} minute [mm] after addition of stimulus.

Statistical evaluation

All experiments were performed in duplicate, and values are expressed as mean ± S.E.M. Statistical analysis was made by Dunnett comparison test after parametric analysis of variance (ANOVA) for repeated measures, and p values below 0.05 were considered significant.

Results

Fig. 1a illustrates the effects of FMLP, A23187, thrombin and PMA on aggregation of blood platelets and PMN leukocytes. Thrombin increased the aggregation of blood platelets to 166.0 ± 3.5 mm, A23187 to 142.9 ± 2.9 mm, and PMA to 84.1 ± 9.8 mm. Aggregation of platelets induced by FMLP was not significantly increased (5.3 ± 0.9 mm) in comparison with control (2.1 ± 0.8 mm).

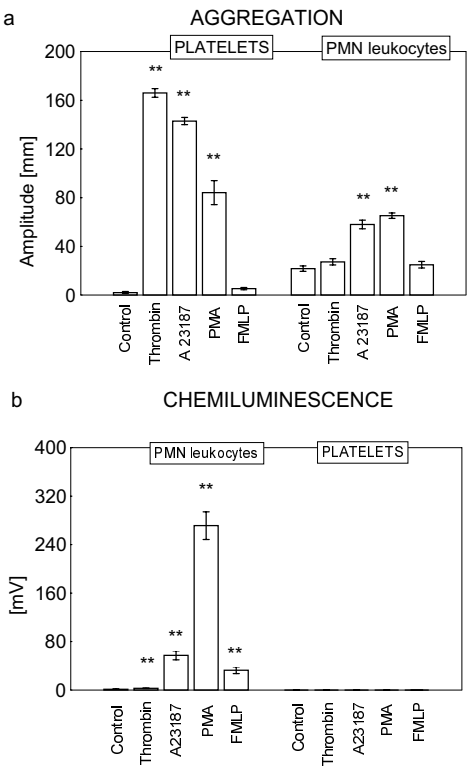

Figure 1. Effects of thrombin, A23187, PMA, and FMLP on a) aggregation of human platelets and PMN leukocytes, b) luminol-amplified chemiluminescence of PMN leukocytes and platelets. Each value represents the mean from 8–10 amplitudes of the aggregation curve at 5^{th} minute [mm] ± S.E.M. or the mean from 8–10 peaks of chemiluminescence [mV] ± S.E.M. * $p < 0.05$, ** $p < 0.01$ vs. control cells.

Aggregation of PMN leukocytes was significantly increased in comparison with control (21.8 ± 2.2) only by A23187 and PMA (to 58.0 ± 3.5 mm and 65.2 ± 2.2 mm, respectively).

Fig. 1b shows chemiluminescence amplified by luminol. None of the stimuli applied induced platelet chemiluminescence. On the other hand, a significant increase of chemiluminescence of PMN leukocytes was observed by PMA, i.e. to 271.4 ± 22.7 mV, which was 5 times more than the stimulation by A23187 (57.4 ± 7.3 mV) and 9 times more than by FMLP (32.9 ± 5.5 mV). Thrombin slightly but significantly increased chemiluminescence from 1.5 ± 0.2 mV (unstimulated PMN leukocytes) to 4.8 ± 1.0 mV.

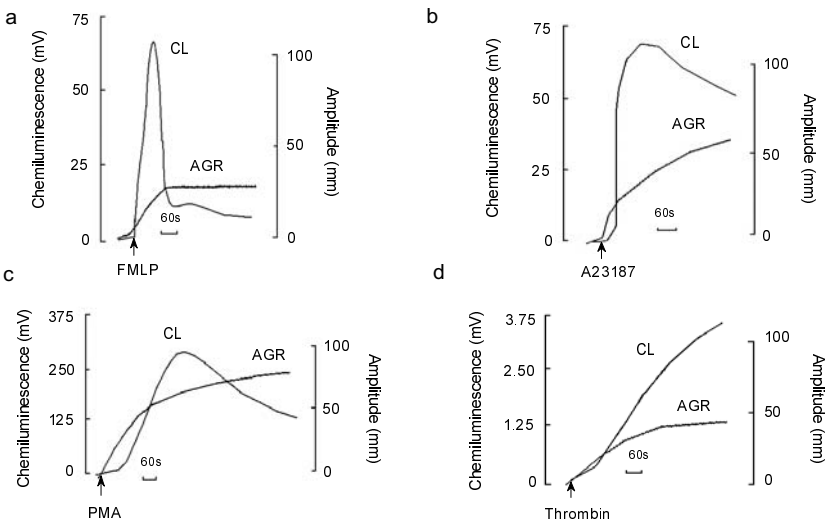

Figure 2. Aggregatory and chemiluminescence curves of PMN leukocytes stimulated with a) FMLP, b) A23187, c) PMA, or d) thrombin. CL – chemiluminescence, AGR – aggregation. Representative curves from 8–10 experiments.

Fig. 2a,b,c,d shows representative curves, time courses and peak responses of aggregation and luminol-amplified chemiluminescence in PMN leukocytes stimulated with FMLP, A23187, PMA, or thrombin, as recorded simultaneously. Maximum chemiluminescence was obtained 1.1 ± 0.1 min after FMLP stimulation, 2.8 ± 0.3 min after A23187 stimulation and 7 ± 0.5 min after PMA stimulation. After thrombin stimulation, the chemiluminescence curve failed to reach a maximum.

On the basis of the results shown in Fig. 1, we investigated interactions between PMN leukocytes and platelets with respect to: (i) aggregation as a response preferential for platelets, (ii) luminol-amplified chemiluminescence as a response typical for PMN leukocytes. Fig. 3 shows the effects of PMN leukocytes on aggregation of blood platelets with respect to different stimuli and two incubation modes. The presence of PMN leukocytes significantly decreased aggregation of thrombin stimulated platelets from 166.7 ± 3.2 mm to 106.5 ± 14 mm (A) and to 101.8 ± 5.5 mm (B), of A23187 stimulated platelets from 137.9 ± 4.3 to 110.1 ± 7.6 mm (A) and to 104.9 ± 7.8 mm (B), and of PMA stimulated platelets from 84.1 ± 9.8 mm to 47.8 ± 2.7 mm (A) and to 48.3 ± 5.6 mm (B). With any stimulus used, the incubation modes A and B failed to exhibit any significant differences in platelet aggregation.

Fig. 4 shows the effects of platelets on FMLP, thrombin, A23187 and PMA induced chemiluminescence of PMN leukocytes. The presence of platelets significantly decreased chemiluminescence from 36.9 ± 9.6 mV to 14.7 ± 4.3 mV (A) and to 8.6 ± 2.0 mV (B) upon stimulation with FMLP, from 3.1 ± 0.4 mV to 2.1 ±

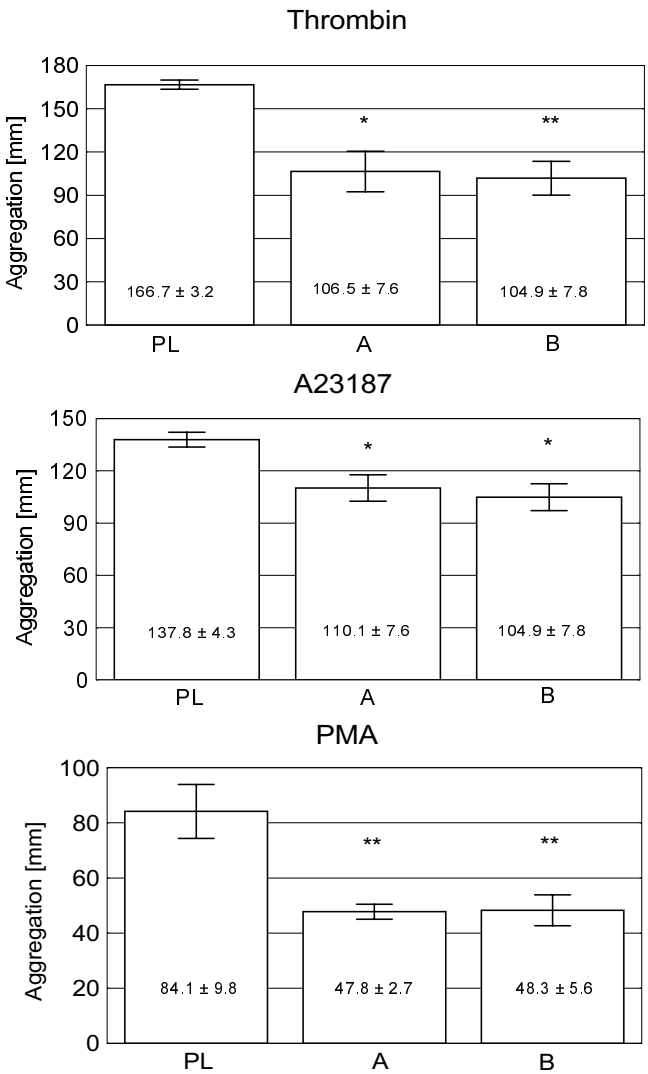

Figure 3. Effects of PMN leukocytes on aggregation of blood platelets with respect to different stimulation and incubation conditions. PMN leukocytes and platelets (1 : 50) were incubated together for 6 min before stimulus (A); PMN leukocytes and platelets were incubated separately and mixed together with the added stimulus (B). Each value represents the mean from 5–6 amplitudes of the aggregation curve at the 5^{th} minute ± S.E.M. * $p < 0.05$, ** $p < 0.01$ *vs.* platelets (PL).

0.3 mV (A) and to 1.7 ± 0.3 mV (B) upon stimulation with thrombin, from 61.2 ± 12.6 mV to 35.5 ± 9.7 mV (A) and to 32.6 ± 6.8 mV (B) upon stimulation with

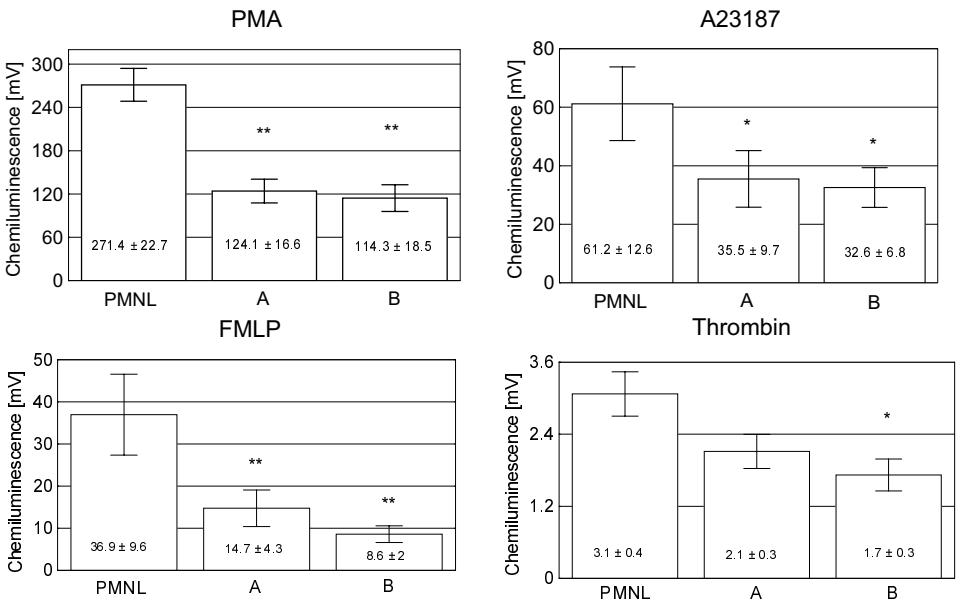

Figure 4. Effects of platelets on luminol amplified chemiluminescence of PMN leukocytes with respect to different stimulation and incubation conditions. PMN leukocytes and platelets (1 : 50) were incubated together for 6 min before stimulus (A); PMN leukocytes and platelets were incubated separately and mixed together with the added stimulus (B). Each value represents the mean from 5–6 peaks of chemiluminescence [mV] ± S.E.M. * $p < 0.05$, ** $p < 0.01$ *vs.* PMN leukocytes (PMNL).

A23187, and from 271 ± 21 to 124 ± 17mV (A) and to 114.3 ± 18.5 mV (B) upon stimulation with PMA. There were no significant differences in chemiluminescence between incubation mode A and B for any stimulus.

Discussion

By means of simultaneous recording of aggregation and chemiluminescence, we studied responses of PMN leukocytes, blood platelets and their mixtures to stimulation by specific as well as nonspecific stimuli for each type of cell. FMLP, a synthetic chemotactic peptide, is a soluble stimulus acting *via* a specific receptor linked to inositol lipid signalling in PMN leukocytes (Heiskanen et al. 1995). The multipotent serine protease thrombin stimulates predominantly platelets through Gi and Gq subunits of platelet membrane G-proteins (Sies 1997). PMA and A23187 bypass receptor-mediated cell signalling pathway. PMA directly activates the intracellular enzyme protein kinase C, while A23187 facilitates the movement of calcium across cellular membranes (Heiskanen and Savolainen 1996; Wang et al. 1997; Ware and Chang 1997).

Under stirring conditions, *in vitro* activation of PMN leukocytes leads to aggregation (Rochon and Frojmovic 1992). Homotypic PMN aggregation depends on the expression and regulation of L-selectin and β_2-integrins, including Mac-1, and the presence of the divalent metals Ca^{2+} and Mg^{2+} in the extracellular milieu is required for aggregation (Lynam et al. 1996). Receptor mediated activation of platelets induced fusion of α-granule membranes to the plasma membrane resulting in the redistribution of P-selectin (Faint 1992). P-selectin, also known as PADGEM, GMP-140 or CD62, is thought to mediate rapid binding of activated platelets to human PMN leukocytes (Hamburger and McEver 1990). The exact mechanism of platelet-leukocyte aggregation is not quite clear but it has been suggested that it may include P-selectin and its ligands CD15 (Hamburger and McEver 1990) and P-selectin glycoprotein ligand-1 (Li et al. 1997), as well as fibrinogen bridging of platelet GPIIb/IIIa and leukocyte CD11/CD18 (Spangenberg et al. 1993).

In our experiments, the non-selective stimuli PMA and A23187 enhanced aggregation of both cell types. We could show that FMLP selective for PMN leukocytes did not activate platelet aggregation but slightly increased aggregation in leukocyte suspensions. Thrombin greatly enhanced the aggregation of platelets, yet aggregation of PMN leukocytes by thrombin was not significantly changed, despite the fact that thrombin can activate some leukocyte populations (Bizios et al. 1987; Hoffman and Church 1993). Thus, aggregation of platelets and PMN leukocytes was increased in the order: PMA < A23187 < thrombin and FMLP < A23187 < PMA, respectively.

When characterizing responses of platelets and PMN leukocytes, aggregation was considered as the preferential response for platelets. The effect of PMN leukocytes on thrombin, A23187 or PMA stimulated aggregation of platelets was studied. Under our experimental conditions, the presence of PMN leukocytes decreased aggregatory responses of platelets to all stimuli applied. The inhibitory effect of PMN leukocytes seemed to be independent of selectivity of the stimulus to the cell, as it was observed in the case of both non-stimulated and stimulated PMN leukocytes. Several *in vitro* studies demonstrated that unstimulated PMN leukocytes could inhibit platelet activation by PMN leukocytes-derived NO, by antiaggregatory metabolites of arachidonic acid and by adenosine (Salvemini et al. 1989; Nicolini and Mehta 1990; Zatta et al. 1990). Additionally, elastase, a PMN-derived protease, was shown to prevent thrombin-induced platelet activation through the cleavage of platelet GP Ib (Cerletti et al. 1992; Aziz et al. 1995). On the other hand, Schattner et al. (1990) suggested that neither arachidonic acid metabolites, reactive oxygen intermediates, nor proteases released by PMN leukocytes were responsible for platelet inhibition. We also demonstrated that cell to cell contact before stimulus addition was not essential for the inhibition of aggregation, as the antiaggregatory effect was identical when cells were first mixed *prior* to stimulation and when they were incubated separately and mixed together with the added stimulus. These findings indicated that the protective effect of PMN leukocytes on platelet aggregation was immediate, occurring within a few seconds. As demonstrated by Valles et al. (1993), the inhibitory effect of PMN on platelet aggregation

was evident in the presence of antibodies against P-selectin, indicating that inhibition of platelet aggregation by neutrophils was enhanced when adhesion was blocked. Thus, neutrophils were more effective in controlling platelet reactivity when the cells were not adhering to each other. It has been suggested that stimulation of platelets in the presence of neutrophils for 60 s resulted in the inhibition of platelet reactivity and P-selectin-induced adhesion step was not required for this down-regulation mechanism (Valles et al. 1993).

Reactive oxygen species production is triggered by particle phagocytosis or by activation by soluble agonists. Firstly, superoxide anion radicals are generated by NADPH-oxidase during respiratory burst. Their dismutation produces hydrogen peroxide. More reactive oxygen species, including hypochlorous acid, hydroxyl radicals, singlet oxygen and peroxynitrite, are derived from reactions of both superoxide radicals and hydrogen peroxide (Heilmann et al. 1995; Mueller and Arnhold 1995). The generation of oxygen metabolites in neutrophils can be evaluated by means of luminol-enhanced chemiluminescence (Dahlgren et al. 1991; Bengtsson et al. 1996). Chemiluminescence of PMN leukocytes increased in the order: thrombin < FMLP < A23187 < PMA. We observed different time courses and peaks of chemiluminescence curve upon stimulation, i.e. a rapid onset of chemiluminescence after FMLP and A23187, and a lag phase for any measurable chemiluminescence after PMA. Since platelets alone did not show detectable chemiluminescence response to any stimulation, we considered luminol amplified chemiluminescence as the dominant response for PMN leukocytes. The effect of platelets on thrombin, FMLP, A23187 or PMA induced chemiluminescence of PMN leukocytes as well as its dependence on the incubation mode before stimulus addition were investigated.

Platelets were found to significantly decrease chemiluminescence of PMN leukocytes induced by all types of stimuli. The inhibitory effect of unstimulated platelets on FMLP-induced chemiluminescence could be associated with an increased generation of neutrophil adenosine. Adenosine enhanced the autoregulatory inhibitory pathway and peripheral accumulation of actin filaments, which form a barrier to extracellular release of reactive oxygen radicals (Bengtsson et al. 1996). Chemiluminescence of PMN leukocytes induced by PMA was significantly decreased by platelets as a result of inhibition of HOCl production, primarily by consuming PMN-derived H_2O_2 *via* their glutathione cycle (Dallegri et al. 1989). Substances released after platelet activation, such as TGF-β, soluble P-selectin, PGDF, adenine nucleotides, may also contribute to the decrease of chemiluminescence (Bazzoni et al. 1992). One of the major constituents of blood platelets, 5-hydroxytryptamine (serotonin) has been shown to scavenge free radical generation (Schuff-Werner et al. 1995). Histamine is produced in platelets exposed to thrombin and PMA and is considered to be an intracellular messenger in platelets (Saxena et al. 1989, 1991). Moreover, histamine from A23187-stimulated platelets is liberated extracellularly independent of the arachidonic acid pathway (Jančinová and Nosáľ 1998). Since histamine has been demonstrated to inhibit superoxide production in PMN leukocytes (Tasaka 1991), this way of platelet contribution to decrease chemiluminescence should be taken into consideration. The results obtained suggest that

the inhibitory effect of platelets on luminol-amplified chemiluminescence may not be dependent on the type of stimulation. Since the stimuli used activated PMN leukocytes by different mechanisms, platelets could either inhibit the common step in oxygen metabolites generation (enzymes involved in activating NADPH oxidase, myeloperoxidase), or interact directly with oxygen radicals.

From our results it is evident that platelets and PMN leukocytes respond in a different way to stimuli when exposed separately or in a mixture. Although further work is needed to elucidate the target sites as well as the mechanisms involved in the inhibitory effects, under defined experimental conditions PMN leukocytes and platelets were found to decrease mutually their dominant responses *in vitro.* The inhibitory effects were dependent either on the selectivity of stimuli, or on cell-to-cell contact before stimulus addition.

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