Inhibition of human platelet activation by polymorphonuclear leukocytes

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- 1 The effect of unstimulated human polymorphonuclear leukocytes (PMNs) on platelet activation was examined.
- 2 Human platelet aggregation and adenosine 5'-triphosphate (ATP) release induced by collagen ($1\text{--}2\text{ }\mu\text{g ml}^{-1}$); thrombin ($0.01\text{--}0.02\text{ u ml}^{-1}$) or arachidonic acid (AA) ($0.1\text{--}0.2\text{ mM}$) were markedly inhibited when conducted in the presence of unstimulated PMNs.
- 3 Platelet inhibition induced by PMNs was dependent on the number of PMNs and on the incubation time of the mixed cell suspension.
- 4 Platelet inhibition was not reversed in time when PMNs were depleted from the mixed-cell suspension.
- 5 PMN-mediated platelet-inhibition was not mediated by AA metabolites, oxygen reactive intermediates, nitric oxide or proteases.
- 6 The factor(s) accounting for the platelet inhibition mediated by PMNs are not yet characterized.

Introduction

Platelet interaction with polymorphonuclear leukocytes (PMNs) occurs at many steps in the inflammatory response, including chemotaxis, adhesion, secretion, degranulation and activation of the respiratory burst (Turner *et al.*, 1977; Marcus *et al.*, 1981; Rhee *et al.*, 1986). Substances that are released by PMNs and which affect platelet behaviour include oxygen radicals (Levine *et al.*, 1986), enzymes (Chignard *et al.*, 1986), arachidonic acid (AA) metabolites (Mehta *et al.*, 1986) and platelet activating factor (PAF) (Lotner *et al.*, 1980).

In a previous study, Zoucas *et al.* (1985) demonstrated that a soluble and filtrable factor(s) released by latex-stimulated PMNs in plasma suspension, impaired platelet aggregation induced by adenosine diphosphate (ADP) and AA. Furthermore, they showed that unstimulated PMNs did not inhibit platelet aggregation. In the present study, we reexamined whether unstimulated PMNs were able to suppress platelet responses.

Methods

Blood samples

Blood samples were obtained from healthy donors who had taken no medication for at least 10 days before the day of sampling. In some experiments, blood samples were obtained from donors who had ingested 500 mg of acetylsalicylic acid (ASA) 24 h before the day of study. Blood was obtained by venepuncture of the forearm vein and it was drawn directly into plastic tubes containing 3.8% sodium citrate (1:9 v/v).

Preparation of polymorphonuclear leukocytes

Peripheral blood PMNs were isolated from citrated human blood samples, as described previously (Geffner *et al.*, 1987) by Ficoll-Hypaque centrifugation and sedimentation in dextran. Contaminating erythrocytes were removed by hypotonic lysis. After washing, the cells were resuspended at a final concentration of $5 \times 10^6\text{ PMNs ml}^{-1}$ in Tyrode albumin buffer (TA buffer). The cell suspension contained 95–98% neutrophils.

Preparation of platelet rich plasma (PRP)

PRP was prepared by centrifugation of the citrated blood samples at 180 *g* for 10 min. The number of cells was adjusted to $5 \times 10^8\text{ ml}^{-1}$ by addition of autologous platelet poor plasma obtained by centrifugation (1500 *g* for 30 min) of the remaining blood sample after removal of the PRP.

Preparation of washed platelets

PRP was centrifuged in the presence of EDTA (5 mM) for 15 min at 1500 *g*. Then, platelets were washed twice (1500 *g* for 20 min) with Tris saline buffer (TS buffer) and finally resuspended in TA buffer at a concentration of $5 \times 10^8\text{ platelets ml}^{-1}$.

Platelet aggregation and adenosine 5'-triphosphate (ATP) release

Platelet aggregation and ATP release were simultaneously measured with a Lumi-aggregometer (Chrono-Log) at 900 r.p.m. and 37°C; $200\text{ }\mu\text{l}$ of PMNs ($5 \times 10^6\text{ ml}^{-1}$) or TA buffer were added to $200\text{ }\mu\text{l}$ of washed platelets (plus 0.5 mg ml^{-1} of fibrinogen) or PRP. When ATP release was measured, $5\text{ }\mu\text{l}$ of luciferin-luciferase (40 mg ml^{-1}) was also added. After 1 min of incubation, platelet agonists were added and aggregation response and ATP release were recorded during 3 min.

In some experiments, mixed-cell suspensions were centrifuged at 160 *g* for 1 min to spin down and eliminate all of the PMNs, as observed by light microscopy.

Materials

Buffers: Tris saline buffer (TS buffer) consisted of: 445 ml of 0.15 M NaCl; 50 ml of Tris HCl 0.15 M, pH = 7.4; 0.5 g of glucose; 10 ml of EDTA 0.10 M and 250 mg of bovine serum albumin (BSA). Tyrode albumin buffer (TA buffer) (pH = 7.4) consisted of (mM): NaCl 137, KCl 2.7, NaHCO_3 11.9, NaPO_4H_2 0.42, MgCl_2 1, CaCl_2 1, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 5, glucose 1 g l^{-1} and BSA 0.35%. PMNs and platelets suspended in TA buffer were used in all the experiments.

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Drugs: the following were used (sources in parentheses) *p*-bromophenacyl bromide (BPB), *N*-tosyl-1-phenylalanine chloromethyl ketone (TPCK), phenylmethanesulphonyl fluoride (PMSF), superoxide dismutase (SOD), catalase, cytochrome C, methaemoglobin, methylene blue, AA (all Sigma), collagen (Hormon-Chemie), and human thrombin (Parke-Davis). Haemoglobin (Sigma) was dissolved in normal saline to produce a 1 mM solution. The haemoglobin was then fully reduced by the method of Martin *et al.* (1985). Briefly, sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) (10 mM) was added to the haemoprotein solution and it was then removed by dialysis against 500 volumes of distilled water for 3 h at 4°C. Aliquots of this solution were then stored at -20°C until used.

Statistics

Results are expressed as mean $\pm$ s.e.mean for *n* separate experiments. Student's paired *t* test was used to determine the significance of differences between means and *P* < 0.05 was taken as statistically significant.

Results

Effect of PMNs on platelet aggregation

The addition of 200 μl of a cell suspension containing PMNs ($5 \times 10^6 \text{ ml}^{-1}$) to 200 μl of washed platelets ($5 \times 10^8 \text{ ml}^{-1}$) completely suppressed the aggregation response to collagen ($1 \mu\text{g ml}^{-1}$) (Figure 1a). The inhibition was completely overcome by increasing the collagen concentration 2–8 fold (Figure 1a). The antiaggregatory activity of PMNs was dependent upon both the number of PMNs in the cell suspension (Figure 1b) and the incubation time of platelets with PMNs (Table 1). Whatever the platelet stimulus employed, 100% inhibition was achieved after 20 min of incubation (Table 1). At this time, platelet-mediated ATP release induced by collagen ($1\text{--}2 \mu\text{g ml}^{-1}$), AA ($0.1\text{--}0.2 \text{ mM}$) or thrombin ($0.01\text{--}0.02 \text{ U ml}^{-1}$) was also completely suppressed (% inhibition = 94 ± 5 , *n* = 3). The suppression of the platelet response induced by PMNs did not require the presence of PMNs during the platelet aggregation assay. In fact, platelets that were preincubated with PMNs and then depleted of them remained inhibited for at least 2 h (Table 2).

Table 1 Time-dependent platelet inhibition induced by polymorphonuclear leucocytes

Agonist	n	Platelet inhibition (%) at different incubation times (min)			
		1	5	10	2
Collagen	5	58 ± 17	93 ± 7	100	100
AA	3	31 ± 9	83 ± 17	91 ± 9	100
Thrombin	3	40 ± 5	55 ± 12	91 ± 9	100

Aliquots (200 μl) of washed platelets ($5 \times 10^8 \text{ ml}^{-1}$) were incubated with 200 μl of PMN suspension ($5 \times 10^6 \text{ ml}^{-1}$) or 200 μl of TA buffer as control samples, for different periods of time at room temperature. Then, platelet aggregation was induced by the addition of collagen, arachidonic acid (AA) or thrombin at concentrations leading under control conditions to 70 to 80% increase in light transmission. These concentrations varied between $1\text{--}2 \mu\text{g ml}^{-1}$, $0.1\text{--}0.2 \text{ mM}$ and $0.01\text{--}0.02 \text{ U ml}^{-1}$ for collagen, AA and thrombin, respectively, according to the donors. The results are expressed as a % inhibition compared to controls. Each value is the mean $\pm$ s.e. of *n* separate experiments.

Table 2 The effect of preincubation and subsequent depletion of polymorphonuclear leukocytes on platelet activation

Treatment	Platelet aggregation (%) at different times (min)		
	0	60	120
Untreated platelets	79 ± 3	75 ± 5	70 ± 4
Centrifuged platelets ^a	71 ± 3	65 ± 3	63 ± 2
Platelet depleted of PMNs by centrifugation ^b	5 ± 2	7 ± 3	10 ± 4

One ml of washed platelets ($5 \times 10^8 \text{ ml}^{-1}$) were incubated with 1 ml of PMNs ($5 \times 10^6 \text{ ml}^{-1}$) (b) or Tyrode albumin buffer (a) for 15 min at room temperature. Then, both cell suspensions were centrifuged at 160 *g* for 1 min. After centrifugation, 800 μl of each supernatant (containing only platelets) was removed and platelet aggregation was induced by collagen ($1\text{--}2 \mu\text{g ml}^{-1}$) at the indicated times. The centrifugation procedure spun down all PMNs, as observed by light microscopy. Untreated platelets represent washed platelets which were not centrifuged. The results are expressed as % of platelet aggregation. Each value is the mean $\pm$ s.e. of 3 separate experiments.

Figure 1 Inhibition of platelet aggregation by polymorphonuclear leukocytes (PMNs). (a) Aliquots (200 μl) of washed platelets ($5 \times 10^8 \text{ ml}^{-1}$) were incubated with 200 μl of PMN suspension ($5 \times 10^6 \text{ ml}^{-1}$) or 200 μl of Tyrode albumin (TA) buffer (control) for 10 min at room temperature. Then, aggregation was induced by the indicated collagen concentrations. Control samples were stimulated by collagen ($1 \mu\text{g ml}^{-1}$). (b) Aliquots (200 μl) of washed platelets ($5 \times 10^8 \text{ ml}^{-1}$) were incubated with 200 μl of PMN suspension containing different PMN concentrations, as indicated in the figure, or with 200 μl of TA buffer (control). After 10 min of incubation at room temperature, platelet aggregation was induced by collagen ($1 \mu\text{g ml}^{-1}$). The figures show typical tracings of aggregation representative of five separate experiments.

Table 3 Effect of some inhibitors on the antiaggregatory activity of polymorphonuclear leukocytes

Treatment		Platelet aggregation (%)		n
		Platelets	Platelets plus PMNs	
None		78 ± 2	35 ± 3	12
ASA*		—	33 ± 4	6
BPB**	10 µM	—	36 ± 6	5
SOD	30 u ml ⁻¹	78 ± 5	16 ± 3	5
Cytochrome C	200 µg ml ⁻¹	82 ± 4	15 ± 3	5
Catalase	1000 u ml ⁻¹	80 ± 4	43 ± 3	5
PMSF	10 µM	74 ± 6	36 ± 5	4
TPCK	10 µM	77 ± 2	43 ± 4	4
FeSO ₄	1 µM	72 ± 4	36 ± 5	4
Methaemoglobin	5 µM	71 ± 4	37 ± 2	4
Haemoglobin	10 µM	63 ± 7	32 ± 6	4
Methylene blue	10 µM	70 ± 4	31 ± 2	4

Aliquots (200 µl) of washed platelets ($5 \times 10^8 \text{ ml}^{-1}$) were incubated with 200 µl of PMN suspension ($5 \times 10^6 \text{ ml}^{-1}$) at room temperature for 1 min, in the presence of the different compounds mentioned above. Then, platelet aggregation was induced by the addition of collagen ($1\text{--}2 \mu\text{g ml}^{-1}$). The results are expressed as % of aggregation. Each value is the mean ± s.e. of *n* separate experiments.

* PMNs were obtained from donors who had ingested 500 mg acetylsalicylic acid (ASA) 24 h before the day of study. Platelets were obtained from donors who had not ingested ASA.

** PMNs were treated with *p*-bromophenacyl bromide (BPB) for 15 min at 37°C. After washing PMNs were added to washed platelets, which were not treated with BPB. Treated vs non-treated: $P < 0.0005$.

The antiaggregatory activity of PMNs was not mediated by a stable releasable factor(s) since supernatants obtained from PMNs or from mixed cell suspensions (PMNs plus platelets) did not modify platelet aggregation induced by collagen ($1\text{--}2 \mu\text{g ml}^{-1}$) ($n = 4$).

The antiaggregatory activity of PMNs was also observed in PRP. When 200 µl of PMNs ($5 \times 10^6 \text{ ml}^{-1}$) were incubated with 200 µl of PRP ($5 \times 10^8 \text{ ml}^{-1}$) for 20 min at room temperature, a marked inhibition of the platelet aggregation response to collagen ($1 \mu\text{g ml}^{-1}$) was observed (% inhibition = 74 ± 6 , $n = 4$).

Mechanisms involved in PMN-mediated platelet inhibition

The activation of phospholipid metabolism and the respiratory burst play an important role in most of the biological

responses mediated by PMNs (Maridonneau-Parini *et al.*, 1986). In order to determine whether any of the metabolites derived from these pathways account for the antiaggregating activity of PMNs, the effect of several inhibitors was assayed.

Since prostacyclin (PGI_2) is a potent platelet inhibitor (Ubatuba *et al.*, 1979), some experiments were done with PMNs obtained from normal donors who have ingested ASA (500 mg) the day before sampling. As shown in Table 3, there were no differences in the inhibitory effect induced by non-treated or ASA-treated PMNs. Moreover, the antiaggregatory activity was not affected by treatment of the PMNs with the phospholipase inhibitor BPB (Blackwell & Flower, 1983) (Table 3).

Superoxide anion and hydrogen peroxide are well known cytotoxic species released by PMNs. The role of these compounds in PMN-induced platelet inhibition was investigated. SOD (30 u ml^{-1}) and cytochrome C ($200 \mu\text{g ml}^{-1}$), two superoxide anion scavengers, were not only unable to decrease the anti-aggregating activity of PMNs but rather they significantly enhanced it (Figure 2 and Table 3). On the other hand, the inhibition of the platelet response was not modified by the hydrogen peroxide scavenger, catalase (Table 3).

To investigate whether the platelet antiaggregatory effect was related to the release of nitric oxide (NO) from PMNs (Rimele *et al.*, 1988), several inhibitory compounds of NO activity were assayed. FeSO_4 ($1 \mu\text{M}$), methaemoglobin ($5 \mu\text{M}$), haemoglobin ($10 \mu\text{M}$) and methylene blue ($10 \mu\text{M}$) were unable to reverse platelet inhibition induced by PMNs (Table 3).

Finally, no loss of antiaggregating activity was observed when the mixed-cell suspensions were incubated with the protease inhibitors TPCK ($10 \mu\text{M}$) and PMSF ($10 \mu\text{M}$) (Kitagawa *et al.*, 1980).

Discussion

In this study, we have demonstrated that PMNs inhibit aggregation of washed human platelets and ATP release induced by several agonists. The antiaggregatory activity of PMNs is dependent on the number of PMNs and on the incubation time of the mixed-cell suspension. In addition, although inhibited platelets do not respond when activated by low agonist concentrations, they normally do at higher ones. Platelet inhibition by PMNs is also observed in platelet-rich-plasma. However, the effect seems to be less effective since the suppression obtained never reached 100%.

Zoucas *et al.* (1985) have previously reported that latex-stimulated PMNs impaired platelet aggregation through the

Figure 2 Platelet inhibition by polymorphonuclear leukocytes (PMNs) is increased by superoxide dismutase (SOD) and cytochrome C. Aliquots (200 µl) of washed platelets ($5 \times 10^8 \text{ ml}^{-1}$) were incubated with 200 µl of PMN suspensions ($5 \times 10^6 \text{ ml}^{-1}$) for 10 min at room temperature in the presence of SOD (30 u ml^{-1}) or cytochrome C ($200 \mu\text{g ml}^{-1}$). Then, aggregation was induced by collagen ($4 \mu\text{g ml}^{-1}$). Control: 200 µl of platelets ($5 \times 10^8 \text{ ml}^{-1}$) were incubated with 200 µl of Tyrode albumin buffer for 10 min at room temperature and then stimulated by collagen ($4 \mu\text{g ml}^{-1}$). The figure shows typical tracings of aggregation representative of five experiments.

release of a soluble factor(s). Moreover, they showed that unstimulated PMNs were unable to inhibit the platelet response. On the contrary, our data demonstrate that unstimulated PMNs markedly suppress platelet aggregation. Controversial results may be explained considering that different experimental conditions were employed in each case. It is important to point out that in the system of Zoucas *et al.* (1985), PMNs were enclosed in dialysis tubes whereas in our experimental conditions, PMNs and platelets were in close cell-to-cell contact. The fact that platelet inhibitory activity is expressed by unstimulated PMNs suggests that this activity is normally present in PMNs. However, we cannot rule out a baseline stimulation of PMNs due to the procedures employed during their purification. Moreover, specific PMN activation by the agonists employed seems not to play a major role in platelet inhibition since platelets preincubated with unstimulated PMNs and then depleted of them remained inhibited when activated by AA, collagen or thrombin.

Our results showing that supernatants from PMNs or PMNs plus platelets do not possess antiaggregatory activity suggest: (1) that there is no PMN releasable factor(s), therefore the inhibition could be related to close cell-to-cell contact; (2) that there is a PMN releasable factor(s) with a very short half life. Interestingly, the presence of PMNs is not permanently required for inhibiting platelet responses since, as was previously mentioned, platelets which were preincubated with PMNs and then depleted of them remained inhibited. Therefore, it could be possible that exposure of platelets to factor(s) released by PMNs or to component(s) of the PMN's membrane are involved in the onset of the reaction.

With regard to the mechanisms implicated in platelet inhibition mediated by PMNs, our results suggest that neither AA metabolites, reactive oxygen intermediates nor proteases released by PMNs are responsible for platelet inhibition. These conclusions are supported by the following findings: (1) PGI₂, which is a very potent platelet inhibitor (Ubatuba *et al.*, 1979)

does not account for the PMN-induced inhibition since similar results were obtained with non-treated and ASA-treated PMNs. Moreover, the treatment of PMNs with BPB, a phospholipase inhibitor (Blackwell & Flower, 1983) did not prevent platelet inhibition; (2) PMSF and TPCK, two protease inhibitors did not modify the inhibitory activity of PMNs and (3) SOD and cytochrome C, two superoxide anion scavengers enhanced the ability of PMNs to inhibit platelet responses while catalase had no effect.

Endothelial derived relaxing factor (EDRF), now characterized as nitric oxide (NO) (Palmer *et al.*, 1987) is a potent vasodilator and platelet inhibitor with a very short half life. Superoxide anions destroy this substance and Fe²⁺, methaemoglobin, haemoglobin and methylene blue interfere with its activity (Gryglewski *et al.*, 1986). Recently, it was indicated that stimulated and non-stimulated PMNs release NO (Rimele *et al.*, 1988). Our results showing that the antiaggregatory activity of PMNs is: (1) enhanced by SOD and cytochrome C; (2) not found in the supernatants of PMNs plus platelets suspensions and (3) more active in washed platelets than in PRP fit with the biological properties described for NO (Moncada *et al.*, 1988). However, NO seems not to be responsible for the antiaggregatory activity of PMNs since it was not reversed by Fe²⁺, methaemoglobin, haemoglobin or methylene blue. Further studies are required to characterize the nature of the inhibitory mechanism(s) involved in this interesting model of cell interaction.

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