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MODULATION BY SUPEROXIDE ANIONS OF NEUTROPHIL-MEDIATED PLATELET ACTIVATION

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Abstract—When polymorphonuclear neutrophil-platelet suspensions were stimulated by 0.5 μM *N*-formyl-Met-Leu-Phe in the presence of 40 U/mL of superoxide dismutase, a significant reduction of platelet secretion was observed (51.4 ± 6.3% vs 62.4 ± 4.6% for control; mean ± SEM; $N = 6$; $P < 0.01$). This was due to the superoxide anion scavenging property of superoxide dismutase since neutrophil degranulation, cathepsin G and elastase enzymatic activities (the two main mediators of this cell-to-cell interaction) and platelet reactivity were not affected. Involvement of superoxide anions was confirmed using leukotriene B_4, a neutrophil agonist which induces degranulation with minimal superoxide anion production. Indeed, serotonin release induced by this agonist was unchanged whether superoxide dismutase was added or not.

Key words: Neutrophil; platelet; superoxide anions

Activation of human platelets induced by stimulated PMN† firstly shown by Chignard *et al.* [1] and confirmed by others [2, 3], has been extensively studied in order to elucidate the mechanisms by which such a cell-to-cell interaction occurs. We have recently demonstrated that the observed platelet response was due to the combined effects of cathepsin G (Cat. G, EC 3.4.21.20) and elastase (HLE, EC 3.4.21.37), two serine proteinases released from activated PMN [4]. However, although Cat. G and HLE are the essential mediators of the PMN-mediated platelet activation, a modulatory effect by other PMN-derived substances may intervene and contribute to either an enhancement or an inhibition of the platelet reactivity. It appears that the role of oxygen metabolites is largely evoked in the literature [5]. In fact, the main product of the oxygen reduction by activated PMN are superoxide anions (O_2^-) which have been described as pro-aggregatory agents [6–8]. Thus, these anions promote platelet aggregation when low concentrations of thrombin were used [6, 7] or can even directly activate platelets [6].

The purpose of the present study was to investigate whether these oxygen metabolites generated by activated PMN also participate in the proteinase-mediated PMN–platelet interaction.

MATERIALS AND METHODS

Materials. Blood from healthy volunteers was obtained from the Centre National de Transfusion Sanguine (Paris, France). Heparin was from Choay (Paris, France) and 4-nitrophenyl-β-D-glucopyranosiduronic acid from Merck (Darmstadt, FRG). BSA was from Euromedex (Strasbourg, France). HEPES, SOD, cytochrome *c*, prostacyclin, dextran, *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide, *N*-succinyl-ala-ala-ala-*p*-nitroanilide, cytochalasin B and fMLP were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Hanks' balanced salt solution (HBSS) was from Gibco (Paisley, U.K.). Ficoll-Paque was obtained from Pharmacia (Uppsala, Sweden). Biodegradable counting scintillant (BCS) and [^{14}C]serotonin were from Amersham International (Amersham, U.K.) and fibrinogen (Grade L) was purchased from Kabi (Stockholm, Sweden) and was treated with diisopropyl fluorophosphate to inactivate coagulant contaminants. Leukotriene B_4 (LTB_4) was obtained from Cascade Biochem LTD (Berkshire, U.K.). Cat. G was purified from human PMN as previously described [4], following the method of Baugh and Travis [9] modified by Martodam *et al.* [10].

Cell purification. Cells were prepared from human blood as previously described [4]. At the end of the purification procedure, [^{14}C]serotonin loaded platelets (4×10^8 cells/mL) were resuspended in Tyrode's buffer (composition, mM: NaCl 137.0; KCl 2.68; $NaHCO_3$ 11.9; NaH_2PO_4 0.42; $CaCl_2$ 2.0; $MgCl_2$ 1.0; glucose 5.5; HEPES 5.0 and BSA 0.35%; pH 7.4). Isolated PMN (10^7 cells/mL) resuspended in HBSS were more than 96% pure and their viability was more than 98%, as determined by Türk's stain and the Trypan blue dye exclusion method, respectively.

Measurement of serotonin release. Washed platelets (2×10^8 cells/mL) and purified PMN (5×10^6 cells/mL) were preincubated in a shaking water bath at 37° with fibrinogen (0.7 mg/mL), $CaCl_2$ (1.3 mM) and $MgCl_2$ (1 mM). Once cytochalasin B (5 μg/mL) was added, SOD (40 U/mL) and either the PMN agonist (fMLP or LTB_4) or the platelet agonist (Cat. G) were added 2 and 5 min later, respectively. The

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† Abbreviations: PMN, polymorphonuclear neutrophils; SOD, superoxide dismutase; O_2^-, superoxide anions; fMLP, *N*-formyl-Met-Leu-Phe; and LTB_4, leukotriene B_4.

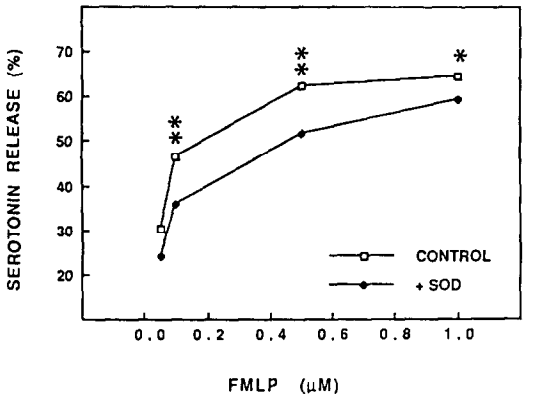

Fig. 1. Effect of superoxide dismutase on platelet serotonin release induced by fMLP-activated PMN. PMN and platelets were incubated together at 37° and stimulated by increasing concentrations of fMLP. SOD (40 U/mL) was added in the test tube 3 min before addition of fMLP. Platelet activation was evaluated by % of serotonin release. Each value is the mean of six distinct experiments. At all points, SEM were smaller than eight for controls and SOD-treated samples.

reaction was stopped 5 min after stimulation by centrifugation (2500 *g*; 10 min; 4°). The [^{14}C]-serotonin release was determined from 400 μL of supernatants mixed with BCS for scintillation counting using a Counter 1212 Rack-beta (LKB, Wallac, Stockholm, Sweden) and expressed in % of the total platelet [^{14}C]serotonin content.

Determination of cathepsin G and elastase enzymatic activities. Cat. G and HLE active site concentrations were evaluated from supernatants of activated PMN processed as for serotonin release determination. Enzymatic activities were recorded by measuring hydrolysis at 410 nm of their specific synthetic substrates, i.e. *N*-succinyl-ala-ala-pro-phe-*p*-nitroanilide and *N*-succinyl-ala-ala-ala-*p*-nitroanilide (1 mM final concentration), respectively.

β-Glucuronidase release determination. According to the experiments, PMN (5×10^6 cells/mL) or PMN and platelets (2×10^8/mL) were preincubated for 5 min with cytochalasin B (5 μg/mL) in a shaking water bath at 37° and stimulated by increasing concentrations of fMLP or LTB_4. The reaction was stopped 5 min later by centrifugation (2500 *g*; 10 min; 4°) and supernatants containing released enzymes were collected and stored at −20°. Pellets were resuspended in Triton X-100 0.2%, incubated at 4° overnight and centrifuged (2500 *g*; 10 min; 4°) in order to obtain the cell-bound fractions of the enzyme. The β-glucuronidase activity was determined spectrophotometrically using 4-nitrophenyl-β-D-glucupyranosiduronic acid as substrate. Results were expressed as the percentage of enzyme released in comparison with the total enzyme content.

Superoxide anion generation assay. Production of O_2^- from PMN treated as for β-glucuronidase determination was evaluated by the superoxide dismutase-inhibitable reduction of cytochrome *c* measured at 550 nm. Cytochrome *c* (100 μM final concentration) was added 1 min before addition of PMN agonists. Amounts of O_2^- produced were calculated using an extinction coefficient of 21.1 cm^{-1} mol^{-1} and expressed in nmol/5 min/5 × 10^6 PMN.

Statistical analysis. The data were analysed with the Statview program (1.0, 1985). Data are presented as the mean ± SEM and compared using a two way analysis of variance. Significant differences between experiments were determined using the paired Student's *t*-test with a threshold of $P < 0.05$ (*) or $P < 0.01$ (**).

RESULTS AND DISCUSSION

In order to investigate a possible involvement of O_2^- in PMN-induced platelet activation, we performed experiments using SOD, an enzyme highly specific for this oxygen metabolite species [11]. PMN-platelet mixed cell suspensions were thus stimulated by increasing concentrations of fMLP, either in presence of SOD (40 U/mL) or not, and subsequent platelet activation was determined by measuring serotonin release. As shown in Fig. 1, upon fMLP challenge, serotonin was secreted in a concentration-dependent manner. As hypothesized, and for concentrations of fMLP of 0.1 μM and above, this release was significantly lowered in the presence of SOD (26.3 ± 8% inhibition for 0.1 μM). This effect was unrelated to a direct effect on platelets since serotonin release triggered by exogenously added

Table 1. Effect of superoxide dismutase on PMN activation

	O_2^- production	β-gluc. release	Cat. G activity	HLE activity
Control	67.9 ± 4.5	32.2 ± 2.9	11.0 ± 0.5	7.8 ± 0.9
+SOD	2.5 ± 0.7	30.5 ± 2.3	10.1 ± 0.7	7.3 ± 1.0
P	<0.01	>0.1	>0.1	>0.1

Four parameters of PMN activation were measured following stimulation by 0.5 μM fMLP in absence (control) or in presence of 40 U/mL SOD (for details see Materials and Methods). Values are expressed as follows: O_2^- production in nmol/5 min/5 × 10^6 PMN, β-glucuronidase release in % of the total content and enzymatic activities of Cat. G and HLE in Δ O.D. × 10^{-3}/min. For each parameter basal values were deduced.
Each data is the mean ± SEM of three to eight distinct experiments.

purified Cat. G was not modified by the presence of this inhibitor (44.4 ± 10% vs 41.6 ± 8% for control; N = 5; P > 0.1). As shown in Table 1, this inhibitory effect was also distinct from a possible reduced activation of PMN, as evaluated by measurement of β-glucuronidase release from azurophilic granules (P > 0.1). Moreover, these results indicated that SOD also failed to affect the secretion of Cat. G and HLE, the two main mediators of this cell-to-cell interaction since they are stored in the same granules as β-glucuronidase [12]. Nonetheless, it was verified that the enzymatic activities of these two proteinases were not modified. In fact, active sites of Cat. G and HLE were present in equal amounts in supernatants of fMLP-stimulated PMN whether SOD was added or not (Table 1).

The most obvious conclusion was that the observed biological effect of SOD was the consequence of the specific destruction of O_2^- produced by activated PMN. Another possible explanation would have been an effect of the O_2^- dismutation product H_2O_2. However, this is quite unlikely since recent studies demonstrated that H_2O_2, as O_2^-, acts as an enhancer of platelet activation [13–15].

In order to reinforce the demonstration of the potential involvement of O_2^- in the PMN-platelet cooperation system, we used LTB_4 in place of fMLP, the former being a less potent inducer than the latter of the O_2^- formation by PMN [16]. As shown in Fig. 2a, under our experimental conditions, O_2^- production induced by LTB_4 was almost negligible with regard to that measured upon fMLP challenge. Indeed, PMN synthetized 70.6 ± 4.6 nmol O_2^-/5 min/5 × 10^6 PMN (N = 4) when stimulated with 1 μM fMLP, as compared with 11.8 ± 1.6 nmol O_2^-/5 min/5 × 10^6 PMN (N = 4) using a similar concentration of LTB_4. Despite its weak efficiency for O_2^- production, LTB_4 was described as an agonist of the PMN-platelet cooperation system [2]. In fact, the difference between fMLP and LTB_4 was less important concerning the degranulation (Fig. 2b) which reached 43.2 ± 2.4% (N = 7) and 31.6 ± 3.9% (N = 4) for 1 μM fMLP and LTB_4, respectively. Thus, SOD was used in the cooperation system triggered by LTB_4, i.e. under conditions in which an O_2^- production was possibly too small to modulate platelet reactivity. As expected, and as clearly illustrated in Fig. 3, this O_2^- scavenger was unable to significantly modify serotonin release in such a PMN-platelet activation system (P > 0.1).

These data demonstrated that O_2^- can intervene in platelet activation induced by fMLP-stimulated PMN. We have previously shown that blockade of Cat. G and HLE by a dual anti-proteinase inhibitor such as eglin C, totally supressed platelet activation [17] indicating that O_2^- alone failed to trigger platelet serotonin release in our experimental conditions. This is consistent with data published by Handin *et al.* [6] who showed that the threshold rate production of O_2^- able to directly activate platelets was of 18 nmol/min. Since in our hands production of O_2^- by PMN stimulated by 1 μM fMLP and susceptible to contact with platelets was only 14.2 ± 1.8 nmol/min, it was too small to be efficient by itself. It thus appears that the participation of O_2^- is limited to an enhancement of platelet activation induced by sub-

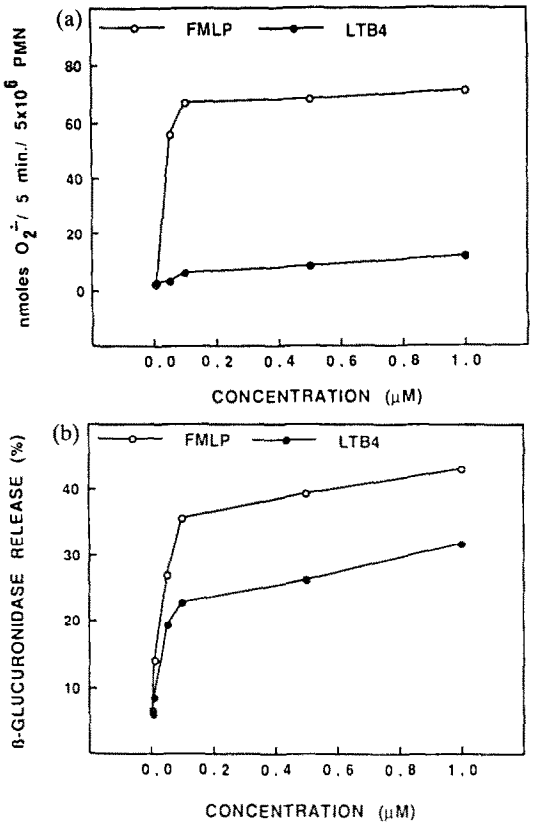

Fig. 2. Comparison of PMN activation induced by fMLP or LTB_4. Two parameters of PMN activation, O_2^- production (Fig. 2a) and β-glucuronidase release (Fig. 2b) were measured from PMN stimulated by fMLP or LTB_4. Each point is the mean of four to eight distinct experiments. At all points, SEM were smaller than 4.6 and 3.9 (Fig. 2a) and than 4.5 and 1.6 (Fig. 2b) for fMLP and LTB_4, respectively.

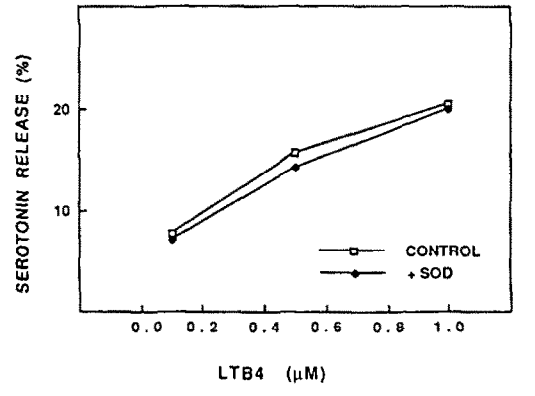

Fig. 3. Effect of superoxide dismutase on platelet serotonin release induced by LTB_4-activated PMN. Same legend as in Fig. 1 using LTB_4 in place of fMLP. At all points, SEM were smaller than 3.4 for controls and 3 for SOD-treated samples.

maximal concentrations of fMLP. This phenomenon occurs apparently only when a certain amount of O_2^- is produced. Indeed, using LTB_4 as agonist, or fMLP at 0.05 μM or less (not shown), there was no significant inhibition by SOD. For this latter concentration of fMLP, 55.5 ± 3.1 nmol O_2^-/5 min/ 5×10^6 PMN were produced (N = 5), this amount being significantly lower ($P < 0.05$) than that measured upon 0.1 μM fMLP challenge (66.6 ± 2.9 nmol O_2^-/5 min/5 × 10^6 PMN; N = 5), a concentration for which a protective effect of SOD was observed.

These data illustrate a new model of striking synergy between proteinases and oxygen metabolites. It is tempting to speculate that such a mechanism could operate *in vivo*. Indeed, it has been shown that under certain experimental conditions, Cat. G activates platelets in the presence of plasma [18] on the one hand, and O_2^- are produced in whole blood [15] on the other hand. Several arguments call for the participation of the PMN-mediated platelet activation in different pathological states such as the adult respiratory distress syndrome. Interestingly, the involvement of serotonin has been described in this disease [19, 20]. It is also of note that administration of SOD has a beneficial effect in shock or post-ischemic injury, two disorders for which a role for PMN and platelets has been evoked [21, 22].

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