Biochemical Pharmacology, Vol. 40, No. 10, pp. 2265–2269, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

INHIBITORY EFFECT OF UNSTIMULATED NEUTROPHILS ON PLATELET AGGREGATION BY RELEASE OF A FACTOR SIMILAR TO ENDOTHELIUM-DERIVED RELAXING FACTOR (EDRF)

FRANCESCA ANTONIA NICOLINI and JAWAHAR LAL MEHTA*
Division of Cardiology, University of Florida College of Medicine and the V.A. Medical Center, Gainesville, FL, U.S.A.

(*Received* 4 *December* 1989; *accepted* 5 *June* 1990)

Abstract—Previous studies have indicated a possible role for polymorphonuclear leukocytes (PMNLs) in the maintenance of hemostasis and vascular tone. We now demonstrate that unstimulated isolated PMNLs maintained at 37° inhibited human platelet aggregation in a concentration- and time-dependent fashion. In addition, PMNLs increased platelet cyclic GMP concentrations. The platelet aggregation inhibitory effect of PMNLs was potentiated by superoxide dismutase and attenuated by hemoglobin and methylene blue. This inhibitory effect of PMNLs was not observed in 48-hr-old killed cells and was not modulated by aspirin treatment or by adenosine deaminase. These observations suggest that human PMNLs maintained at 37° produce a substance with biological characteristics similar to those of the endothelium-derived relaxing factor.

Several investigators have suggested that leukocytes, in particular polymorphonuclear leukocytes (PMNLs), play an important role in the maintenance of hemostasis and vascular tone [1–7]. PMNLs appear in the thrombus early in the formation of a clot and may participate in non-plasmin-mediated thrombolysis [1]. PMNLs also inhibit platelet activation by the release of elastase [2]. On the other hand, oxygen-derived free radicals released from activated PMNLs stimulate platelet aggregation and release reaction [3, 4], and may have a role in platelet-induced thrombosis. PMNLs also participate in tissue injury and in the extension of myocardial damage following acute coronary occlusion and reperfusion [5, 6]. We have shown recently an important smooth muscle relaxant effect of unstimulated human PMNLs mediated via release of a relaxing factor which is qualitatively similar to endothelium-derived relaxing factor (EDRF) [7]. In the present study, we examined the findings of unstimulated PMNLs on human platelet aggregation. Our findings indicate that PMNLs inhibit platelet aggregation in a concentration- and time-dependent fashion and that the inhibition of platelet aggregation may be via release of the same factor that relaxes vascular smooth muscle.

MATERIALS AND METHODS

Blood collection. Peripheral venous blood was collected from normal, healthy volunteers in 3.8% sodium citrate (9:1; v/v) for separation of platelet-rich plasma (PRP). Blood was also collected in heparin (10 units/mL) for separation of PMNLs. None of the volunteers had taken any drugs over the 10 days prior to blood donation.

Preparation of PRP, PPP and PMNLs. To obtain PRP, blood was centrifuged at 150 *g* at 25° for 10 min. The remaining blood was centrifuged at 1200 *g* for 15 min to obtain platelet-poor plasma (PPP). Platelet count in PRP was adjusted to approximately 300,000 per mm^3. Heparinized blood was layered over 3 mL of mono-poly resolving medium (Ficoll-hypaque, sp. g. 1.114) and centrifuged at 500 *g* at 25° for 30 min to obtain PMNLs. The PMNL-rich layer was separated and diluted with an equal volume of Hanks' Buffered Salt Solution (HBSS) without Ca^{2+}, Mg^{2+} but containing 0.1% bovine serum albumin and then centrifuged at 800 *g* at 25° for 15 min. Red blood cells were lysed by briefly (20 sec) suspending the PMNL-rich layer in hypotonic saline solution. Then the PMNL-rich layer was removed and washed with HBSS with Ca^{2+} and Mg^{2+} by centrifuging at 800 *g* at 4° for 15 min. PMNLs were suspended in HBSS containing Ca^{2+} and Mg^{2+}, and the suspension (5×10^7 cells/mL) consistently contained more than 98% PMNLs and their viability, as determined by trypan blue exclusion, was more than 95%.

Platelet aggregation. Platelet aggregation was performed in a dual channel aggregometer (Chronolog, model 540) and recorded on a Fisher 5000 chart recorder. Platelet aggregation was induced with adenosine diphosphate (ADP) used in threshold concentration, which varied from 2 to 5 μM. PRP (± PMNLs) were stirred continuously at 700 rpm. In each experiment, the ADP threshold was kept constant. PRP aggregation was checked repeatedly to ensure that threshold concentration did not change throughout the experiment.

Experimental protocols. PMNLs were incubated with PRP at 37° in concentrations ranging from 1×10^6 to 1×10^7/mL, prior to addition of ADP.

* Address reprint requests to: J. L. Mehta, M.D., Ph.D., University of Florida, College of Medicine, Box J-277, JHMHC, Gainesville, FL 32610.

In other experiments, the concentration of PMNLs (5×10^6/mL) in PRP was kept constant, but the incubation time with PRP was varied from 1 to 60 min.

To determine the mechanism of the effects of unstimulated PMNLs on platelet aggregation, PMNLs were incubated with superoxide radical scavenger superoxide dismutase (SOD, 50 μg/mL), hemoglobin (1 μM), methylene blue (10 μM), adenosine deaminase (5 units/mL), or catalase (1000 units/mL). Each agent was incubated with PMNLs at 37° before their suspension in PRP and addition of ADP to induce platelet aggregation. In five experiments, PMNLs were taken from individuals who were given aspirin (650 mg) by mouth 12 hr earlier, but the PRP was taken from subjects who had not taken aspirin.

To determine if the effects of PMNLs on platelet aggregation were due to physical properties of PMNLs, 48-hr-old PMNLs (40% alive), and killed PMNLs (heated at 50° for 60 min or treated with 100% ethanol), were incubated with PRP. In other experiments, aliquots of the supernatant from 1×10^7 fresh PMNLs/mL were incubated with PRP at 37° for 30 min before addition of ADP to initiate platelet aggregation.

Measurements of thromboxane B_2 (TxB_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and cyclic GMP concentrations. Immediately after platelet aggregation, indomethacin (5 mM) and EDTA (30 mM) were added to the PRP (± PMNLs) suspension to stop the metabolism of arachidonic acid. PRP was centrifuged at 1200 *g* for 15 min at 4° and the supernatants were stored at −70°. On the day of the assay, the supernatants were acidified, extracted with ethyl acetate, and dried under nitrogen. TxB_2 and 6-keto-$PGF_{1\alpha}$ were measured by radioimmunoassay (RIA) in the extracted samples. The minimum reliable detection limits of TxB_2 and 6-keto-$PGF_{1\alpha}$ is 100 pg/mL in this assay [8]. To measure cyclic GMP, 1 mL of 10% trichloroacetic acid was added to PRP at 5 min after onset of aggregation. The PRP (± PMNLs) suspension was then homogenized to disrupt the platelet membranes and extracted with ether three times. The residue was acidified with acetic anhydride, and cyclic GMP was measured by RIA in the extracted samples. The extraction efficiency for this method was 90 ± 5% (N = 12). The supplies for RIA were obtained from New England Nuclear, Boston, MA.

Measurement of adenosine levels. To determine if adenosine is released by PMNLs when in contact with PRP, aliquots of PMNLs (1×10^7/mL) alone and of PRP plus PMNLs both incubated together for 30 min were collected. The samples were then centrifuged at 1200 *g* and frozen at −70°. Adenosine content was measured using HPLC with the detector set to measure adenosine peaks at a wavelength of 254 nm. The methodology for measurement of adenosine is described elsewhere [9]. The detection limit of the assay is 10^{-8} M.

Supplies. Mono-poly resolving medium and HBSS were obtained from Flow Laboratories, Inc., McLean, VA. Bovine serum albumin, human oxyhemoglobin, adenosine deaminase, methylene blue and trypan blue were purchased from the Sigma Chemical Co., St. Louis, MO. Human recombinant SOD (activity: 4000 units/mg) was obtained from Grunenthal GmbH, Stolberg, Federal Republic of Germany.

Statistical analysis. Statistical comparison of the differences between platelet aggregation in PRP alone and the effects of various PMNL concentrations incubated for different times was made by Student's *t*-test. The effects of various modulators of PMNL function were analyzed by analysis of variance and *t*-test, as appropriate.

RESULTS

PMNL-induced inhibition of human platelet aggregation. PMNLs inhibited ADP-induced platelet aggregation in all subjects. The inhibition of platelet aggregation was dependent on the concentration of PMNLs in PRP. In addition, the inhibition of platelet aggregation was dependent on the period of incubation of PRP with PMNLs (Table 1, Fig. 1). A high concentration of PMNLs (1×10^7/mL) incubated for 30–60 min with PRP caused total inhibition of platelet aggregation. ADP alone (up to 10 μM) had no effect on PMNL aggregation or in other PMNL functional parameters, such as chemotaxis or oxygen-derived free radical generation.

Nature of the PMNL-derived inhibitory substance. SOD in nine of ten experiments enhanced ($P < 0.01$) the inhibitory effect of PMNLs on platelet aggregation (Fig. 2). This effect of SOD was observed more consistently when SOD was added to the PRP just prior to addition of PMNLs. When SOD was incubated with PMNLs for 30 min or longer, the potentiation of the platelet aggregation inhibitory effect of PMNLs was less marked. The inhibitory effect of PMNLs was attenuated consistently when oxyhemoglobin and methylene blue were incubated with PMNLs for 30 min ($P < 0.01$) (Table 2). In all these experiments, SOD, oxyhemoglobin or methylene blue had no effect on PRP aggregation in the concentration which modulated the effects of PMNLs. Adenosine deaminase or catalase did not affect aggregation of platelets whether or not PMNLs were present. Furthermore, PMNLs from aspirin-treated subjects inhibited platelet aggregation in the same fashion as those from non-aspirin-treated subjects (data not shown).

Forty-eight-hour-old PMNLs did not inhibit platelet aggregation. Similarly, killed PMNLs or PMNL supernatants (separated within 5 min) had no effect on platelet aggregation. In all these experiments, fresh PMNLs caused marked inhibition of platelet aggregation (Table 3).

TxB_2, 6-keto-$PGF_{1\alpha}$, cyclic GMP and adenosine concentrations. TxB_2 was not affected by the addition of PMNLs to PRP (6.1 ± 1.7 vs 4.3 ± 0.9 ng/mL; P = NS). 6-keto-$PGF_{1\alpha}$ was below the detection limit in all PMNL supernatants and in PRP plus PMNL aliquots. Cyclic GMP increased in PRP in response to 1×10^7 PMNLs (0.149 ± 0.010 vs 0.090 ± 0.008 pmol/100 μL; $P < 0.02$). Adenosine was not detected in either the PMNL supernatants or the PRP plus PMNL suspension.

Table 1. Inhibition of human platelet aggregation by PMNLs

	Platelet aggregation (%)
Concentration-dependence	
PRP alone	69 ± 13 (8)
+10^6 PMNLs/mL	50 ± 8* (6)
+5 × 10^6 PMNLs/mL	35 ± 15† (7)
+10^7 PMNLs/mL	24 ± 10† (5)
Time-dependence	
PRP alone	67 ± 7 (5)
+5 × 10^6 PMNLs/mL (Inc. time: 1 min)	52 ± 7* (5)
+5 × 10^6 PMNLs/mL (Inc. time: 30 min)	37 ± 7† (4)
+5 × 10^6 PMNLs/mL (Inc. time: 60 min)	26 ± 6† (4)

Values are means ± SD; numbers in parentheses are the number of experiments. Inc. = incubation.

* $P < 0.05$, when compared with PRP alone.

† $P < 0.01$, when compared with PRP alone.

Table 2. Modulation of the effects of PMNLs on human platelet aggregation by SOD, adenosine deaminase, hemoglobin and methylene blue

	Platelet aggregation (%)	Inhibition (%)
PRP alone	61 ± 6*	
+PMNLs	42 ± 9	31
+SOD	59 ± 7	
+SOD + PMNLs	29 ± 11	51†
PRP alone	60 ± 9	
+PMNLs	34 ± 8	44
+Oxyhemoglobin	61 ± 9	
+Oxyhemoglobin + PMNLs	50 ± 13	18†
PRP alone	68 ± 6	
+PMNLs	41 ± 6	40
+Methylene blue	65 ± 4	
+Methylene blue + PMNLs	54 ± 11	17†
PRP alone	62 ± 12	
+PMNLs	36 ± 13	41
+Adenosine deaminase	66 ± 8	
+Adenosine deaminase + PMNLs	39 ± 9	41

The concentration of PMNLs was 5 × 10^6/mL in each experiment. Concentrations of SOD, oxyhemoglobin, methylene blue and adenosine deaminase were 50 μg/mL, 1 μM, 10 μM and 5 units/mL respectively.

* Values are means ± SD, N = 4–10 experiments.

† $P < 0.01$ vs inhibition caused by PMNLs.

Table 3. Effect of old and killed PMNLs, and PMNL supernatant on human platelet aggregation

	Platelet aggregation (%)
PRP alone	57 ± 5 (9)
+5 × 10^6 Fresh PMNLs/mL	32 ± 4* (6)
+5 × 10^6 48-hr-old PMNLs/mL	57 ± 3 (4)
+5 × 10^6 killed PMNLs/mL	41 ± 11 (5)
+Supernatant fresh PMNLs (1 × 10^7/mL)	55 ± 5 (5)

Values are means ± SD; numbers in parentheses are the number of experiments.

* $P < 0.001$ vs PRP alone.

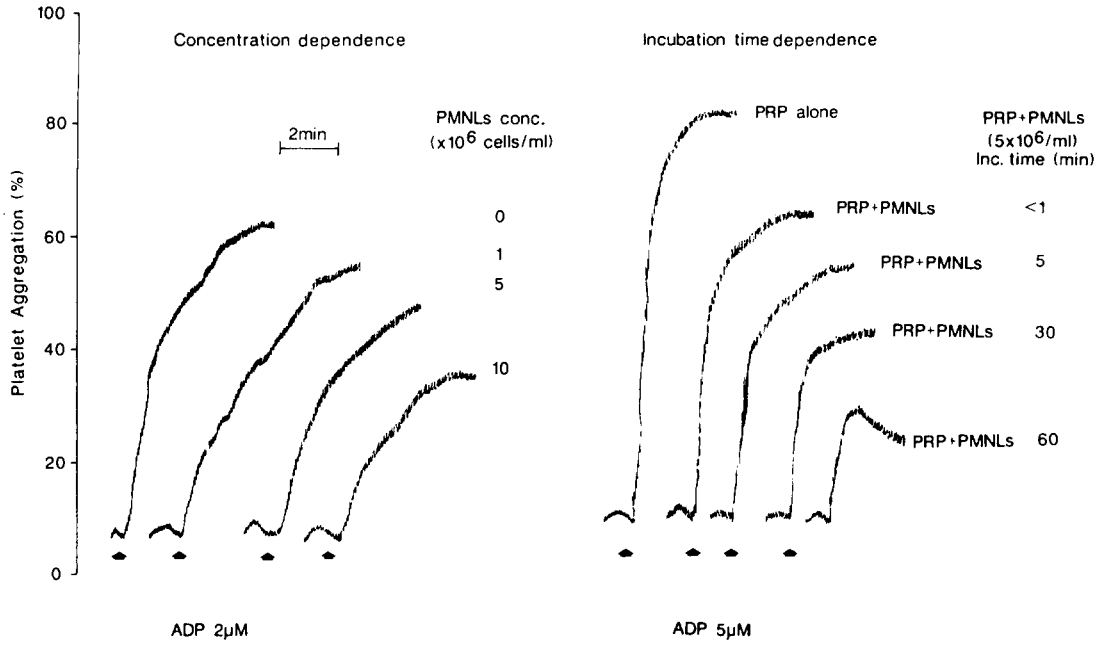

Fig. 1. Inhibition of platelet aggregation by PMNLs in concentration-dependent (left) and incubation time-dependent (right) fashion in a representative experiment.

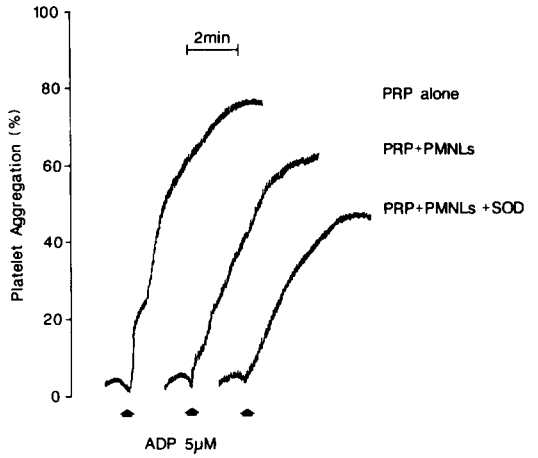

Fig. 2. Potentiation of inhibitory effects of PMNLs on platelet aggregation by SOD.

DISCUSSION

This study indicates that unstimulated human PMNLs inhibit ADP-induced platelet aggregation in PRP in a concentration-dependent fashion. The inhibitory effect of PMNLs was proportional to the duration of contact between PMNLs and PRP at 37°. Furthermore, SOD significantly potentiated the inhibitory effects of PMNLs on platelet aggregation, whereas both oxyhemoglobin and methylene blue attenuated this effect. Our studies also show that the inhibition of platelet aggregation by PMNLs was not related to the release of platelet inhibitors, adenosine and prostaglandin I_2 (PGI_2), from PMNLs, since the treatment of PMNLs with adenosine deaminase or use of PMNLs from aspirin-treated subjects did not modify the aggregation inhibitory effects of PMNLs. Furthermore, adenosine or 6-keto-$PGF_{1\alpha}$ was not detectable in the PMNL supernatant.

The potentiation of activity of the aggregation inhibitory factor from PMNLs by SOD was more pronounced when SOD was present in the incubate for a short period (less than 5 min). Since SOD has a relatively short half-life [10], it is possible that SOD when present in the incubate for long periods loses its efficacy. Potentiation of the bioactivity of PMNL-released aggregation inhibitory factor by SOD suggests that this factor may be degraded by superoxide radicals. Inhibition of the aggregation inhibitory effects of PMNLs by oxyhemoglobin or methylene blue, both of which inhibit intracellular increase in cyclic GMP [11], suggests that the aggregation inhibitory factor may act by increasing cyclic GMP levels. Indeed, our studies show that PMNLs cause an increase in cyclic GMP in PRP.

In our previous studies, we showed that unstimulated human PMNLs relax vascular smooth muscle, and this relaxant effect is potentiated by SOD and captopril, and attenuated by oxyhemoglobin and methylene blue [7]. In this context, it appears that the biological effects of PMNLs on human platelets are related to the release of the same smooth muscle relaxing factor. EDRF has also been identified to inhibit platelet aggregation and relax smooth muscle [12]. These effects of EDRF are potentiated by SOD and inhibited by oxyhemoglobin and methylene blue [11]. Taken together, these observations indicate that the PMNL-derived relaxing factor is qualitatively similar to the EDRF.

A recent study indicates that human PMNLs generate nitric oxide when incubated at 37° [13]. EDRF

has also been identified to be nitric oxide, derived from the amino acid *l*-arginine [14]. Interestingly, stimulation of PMNLs with serum opsonized zymosan, formyl-methionyl-leucine-phenylalanine (f-MLP) or phorbol myristate acetate (PMA), while inducing respiratory burst and lysosomal release, causes no additional nitric oxide production [13].

These studies indicate that unstimulated human PMNLs inhibit platelet aggregation in PRP. Similar observations have been made by Salvemini *et al.* [15] in washed platelet suspension with thrombin as stimulus for platelet aggregation. Our studies extend these observations in human PRP. We now also show that the inhibition of platelet aggregation was not due to release of prostaglandins or adenosine from the PMNLs. In addition, the degree of inhibition of platelet aggregation seems to be dependent on the duration of incubation of PMNLs and PRP. Although elastase release from PMNLs may inhibit platelet aggregation [2], it is unlikely that a significant amount of elastase is released by unstimulated PMNLs to inhibit platelets. The most likely nature of the inhibitory factor based on the biological characteristics appears to be nitric oxide.

Acknowledgements—The authors wish to thank LaVerne A. Randolph for her expert secretarial assistance in the preparation of this manuscript. This work was supported by a Merit Review award from the Veterans Administration, a Veterans Administration Clinical Investigator award to J. L. Mehta, and funds from the American Heart Association, Florida Affiliate.

REFERENCES

1. Moroz LA, Non plasmin-mediated fibrinolysis. *Semin Thromb Hemost* **10**: 80–88, 1984.
2. Brower MS, Levin RI and Garry K, Human neutrophil elastase modulates platelet function by limited proteolysis of membrane glycoproteins. *J Clin Invest* **75**: 657–666, 1985.
3. Handin RI, Karabin R and Boxer GJ, Enhancement of platelet function by superoxide anions. *J Clin Invest* **59**: 959–965, 1977.
4. Canoso RT, Rodview R, Scoon K and Levine PH, Hydrogen peroxide and platelet function. *Blood* **43**: 645–656, 1974.
5. Engler RL, Schmid-Schoenbein GW and Pavala R, Leukocyte capillary plugging in myocardial ischemia and reperfusion in the dog. *Am J Pathol* **111**: 98–111, 1983.
6. Werns SW, Shea MJ and Luccesi BR, Free radicals and myocardial injury: Pharmacologic implication. *Circulation* **74**: 1–5, 1986.
7. Mehta JL, Lawson DL, Nichols WW and Mehta P, Modulation of vascular tone by neutrophils: Dependence on endothelial integrity. *Am J Physiol* **257**: H1315-1320, 1989.
8. Mehta JL, Lawson DL and Mehta P, Modulation of human superoxide production by selective thromboxane synthetase inhibitor U63,557A. *Life Sci* **43**: 923–928, 1988.
9. Findley LJ, Boykin M, Fellon T and Belardinelli L, Plasma adenosine and hypoxemia in patients with sleep apnea. *J Appl Physiol* **64**: 556–561, 1988.
10. Richard VJ, Murry CE, Jennings RB and Reimer KA, Therapy to reduce free radicals during early reperfusion does not limit the size of myocardial infarcts caused by 90 minutes of ischemia in dogs. *Circulation* **78**: 473–480, 1988.
11. Martin W, Villani GM, Jothianandan D and Furchgott RF, Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and methylene blue in the rabbit aorta. *J Pharmacol Exp Ther* **232**: 708–716, 1985.
12. Vanhoutte PM and Rimele TJ, Role of endothelium in the control of vascular smooth function. *J Physiol (Paris)* **78**: 681–686, 1982–1983.
13. Wright CD, Mulsch A, Busse R and Osvald H, Generation of nitric oxide by human neutrophils. *Biochem Biophys Res Commun* **160**: 813–819, 1989.
14. Palmer RMJ, Ashton DS and Moncada S, Vascular endothelial cells synthetize nitric oxide from *l*-arginine. *Nature* **333**: 664–666, 1988.
15. Salvemini D, deNucci G, Gryglewski RJ and Vane JR, Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc Natl Acad Sci USA* **86**: 6328–6332, 1989.