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Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor

(cyclic GMP/arginine/N^G-monomethyl-L-arginine/superoxide dismutase/endothelium-derived relaxing factor)

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ABSTRACT **Incubation of neutrophils or mononuclear cells with washed platelets (all prepared from human venous blood) resulted in an inhibition of thrombin-induced platelet aggregation that was dependent on the number of nucleated cells added. The inhibition was potentiated by superoxide dismutase and reversed by oxyhemoglobin. In the case of neutrophils the inhibition was associated with an increase in cGMP, whereas with mononuclear cells both cAMP and cGMP were increased. The inhibitory activity of neutrophils or mononuclear cells was prevented by their preincubation with N^G-monomethyl-L-arginine methyl ester. L-Arginine reversed the action of N^G-monomethyl-L-arginine methyl ester, whereas D-arginine was ineffective. Preincubation of the cells with catalase or mannitol did not prevent their inhibitory action on platelet aggregation. The inhibition of platelet aggregation was not due to platelet damage or to uptake of thrombin by neutrophils or mononuclear cells. It was overcome by increasing the concentration of thrombin and was absent in cell-free supernatants obtained from a suspension of neutrophils or mononuclear cells or from mixtures of platelets with neutrophils or platelets with mononuclear cells. These data provide evidence for the release of a nitric oxide-like factor from human neutrophils and mononuclear cells. In addition, evidence is provided that, as in stimulated murine macrophages and endothelial cells, the precursor of this factor is L-arginine.**

Activated leukocytes release factors such as superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), or hydroxy acids that increase (1–4) or decrease (5, 6) platelet aggregation induced by several agonists. Harrison *et al.* (7) demonstrated that human neutrophils inhibited platelet aggregation in platelet-rich plasma but erythrocytes did not. Furthermore, Rimele *et al.* (8) demonstrated that rat peritoneal leukocytes elicited by oyster glycogen released a substance that relaxed endothelium-denuded strips of rat aorta. This "neutrophil-derived relaxing factor" (NDRF) had a pharmacological profile similar to that of endothelium-derived relaxing factor (EDRF). EDRF, which is released from vascular endothelium by a variety of agonists, induces vasodilatation (9) and inhibits platelet aggregation (10–12) and platelet adhesion (13, 14) through stimulation of guanylate cyclase. The identification of EDRF as nitric oxide (NO; refs. 15 and 16) explains the stimulation of guanylate cyclase by EDRF, for the enzyme is activated by NO (17). Murine macrophages generate nitrites and nitrates from the guanidino group of L-arginine (18, 19) and a similar pathway for the release of NO has been shown in endothelial cells (20). Indeed, NO is an intermediate to nitrite and nitrate formation in macrophages (21). Here we show that freshly prepared human neutrophils and mononuclear cells release a factor that inhibits platelet aggregation and has a pharmacological profile similar to that described for EDRF (NO). Some of this work was presented to the Physiological Society (22).

MATERIALS AND METHODS

Materials. The Krebs' solution was 137 mM NaCl/2.7 mM KCl/11.9 mM $NaHCO_3$/0.3 mM NaH_2PO_4/0.8 mM $MgSO_4$/5.6 mM glucose/1 mM $CaCl_2$. The cell culture medium consisted of RPMI-1640 medium supplemented with L-glutamine (250 μg/ml), penicillin (85 units/ml), streptomycin (85 μg/ml), and gentamicin (85 μg/ml). Human thrombin, hemoglobin (from bovine blood), superoxide dismutase (from bovine erythrocytes), catalase, mannitol, sodium hydrosulfite, L-arginine (free base), D-arginine (free base), and indomethacin were obtained from Sigma. 3-Isobutyl-1-methylxanthine was obtained from Aldrich. Kits for radioimmunoassay of guanosine 3′,5′-(cyclic) monophosphate (cGMP) and adenosine 3′,5′-(cyclic) monophosphate (cAMP) used cGMP or cAMP labeled at the 2′ position with succinyl-3-[^{125}I]iodotyrosine methyl ester and were purchased from Amersham. Hepes buffer, L-glutamine, penicillin, streptomycin, and gentamicin were obtained from Flow Laboratories. Fetal bovine serum and Hanks' balanced salts solution were obtained from GIBCO. The human monocytic cell line U-937 was obtained from the European Collection of Animal Cell Culture (Salisbury, U.K.). N^G-Monomethyl-L-arginine methyl ester was a gift from Italfarmaco (Milan). Prostacyclin was a gift from Wellcome and iloprost was a gift from Schering.

Preparation of Oxyhemoglobin. Oxyhemoglobin (HbO_2) was prepared by reduction of bovine hemoglobin with sodium hydrosulfite followed by gel filtration with a prepacked disposable column (PD-10, Pharmacia) previously equilibrated with 50 mM Tris/HCl at pH 7.4. The concentration of HbO_2 was determined by a spectrophotometric method (Shimadzu UV-160; A_{576} = 15.99 $mM^{-1} \cdot cm^{-1}$; ref. 23).

Preparation of Washed Platelets. Blood from healthy volunteers who had not taken drugs for at least 15 days was collected by venopuncture into a plastic flask containing 3.15% sodium citrate. Platelet-rich plasma was prepared by centrifugation at 800 × *g* for 8 min. To prepare washed platelets, prostacyclin (300 ng/ml) was then added to the platelet-rich plasma and the mixture was centrifuged at 800 × *g* for 18 min. The supernatant was removed and the platelet pellet was resuspended in 10–15 ml of calcium-free oxygenated (95% O_2/5% CO_2) Krebs' buffer and diluted to 50 ml with the same buffer. Prostacyclin (300 ng/ml) was again added

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Abbreviations: EDRF, endothelium-derived relaxing factor; HHBSS, Hepes-buffered Hanks' balanced salts solution (without Ca^{2+} and Mg^{2+}).

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and the platelets were centrifuged at 800 × *g* for 18 min. The supernatant was aspirated and the pellet was resuspended in calcium-free Krebs' solution (24).

Indomethacin (10 μM, to inhibit the formation of cyclooxygenase products) and Ca^{2+} (1 mM) were added to the final platelet suspension. The platelet count as determined by Coulter Counter was in the range of 1–2 × 10^8 cells per ml.

Isolation of Human Neutrophils and Mononuclear Cells. After removal of the platelet-rich plasma, the buffy coat at the plasma–erythrocyte interface was layered onto a Ficoll-Hypaque density gradient (density 1.095 g/ml) and centrifuged at 200 × *g* for 30 min. This separated mononuclear and polymorphonuclear cells into two distinct bands (25). Contaminating erythrocytes were lysed by the addition of distilled water. Both cell populations were then resuspended in Hanks' balanced salts solution (without Ca^{2+} and Mg^{2+}) containing 10 mM Hepes (HHBSS) and 10 μM indomethacin at pH 7.4. The final leukocyte count was adjusted to 10^8 cells per ml.

Human monocyte-like cells (U-937) were grown in RPMI-1640 medium containing 10% fetal bovine serum. After centrifugation at 1000 rpm for 5 min the cell pellet was resuspended in HHBSS containing 10 μM indomethacin and the volume was adjusted to give a final concentration of 10^8 cells per ml. All cell viability was >95% as judged by trypan blue exclusion.

Measurement of Platelet Aggregation. A suspension of washed platelets (500 μl) was incubated at 37°C for 2 min in a Payton dual-channel aggregometer (26) with continuous stirring at 1000 rpm and then stimulated with submaximal concentrations of thrombin (20–80 milliunits/ml). The decrease in optical density was recorded for 3 min after thrombin stimulation.

Neutrophils, mononuclear cells, or U-937 cells in a volume of 5–60 μl were added to the platelet suspension and the incubation continued for a further 1 min before stimulation with a submaximal concentration of thrombin. Cell numbers added were in the range of 0.5–6 × 10^6. Supernatants from suspensions of neutrophils, mononuclear cells, or their mixtures with platelets, obtained by centrifugation at 10,000 × *g* for 1 min, were also tested at this stage. The supernatants were removed from the pellets of cells and used immediately. When required, catalase, mannitol, superoxide dismutase, or HbO_2 was added to the platelet suspension immediately before the addition of the nucleated cells. The arginine analogue N^G-monomethyl-L-arginine methyl ester was preincubated with neutrophils or mononuclear cells for 50 min before these cells were added to the platelet suspension.

Uptake of Thrombin by Neutrophils and Mononuclear Cells. A suspension of neutrophils or mononuclear cells in Krebs' buffer was incubated for 2 min with thrombin and then centrifuged at 10,000 × *g* for 1 min. Platelet-aggregatory activity of this supernatant was compared with the aggregatory activity of thrombin at the same dilution as that added to the suspension of nucleated white cells.

Measurement of Cyclic Nucleotides. Concentrations of cGMP or cAMP were measured by means of an acetylated iodinated radioimmunoassay kit. A suspension of washed platelets or an equivalent volume of Krebs' solution (500 μl) was preincubated in an aggregometer for 1 min with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (2 μM). Neutrophils or mononuclear cells (or Krebs' solution) were then added and the incubation continued for a further 1 min. Ice-cold trichloroacetic acid (10%, wt/vol) was added (500 μl), the samples were stored at −20°C, and the cyclic nucleotides were extracted as described (27). All determinations were performed in duplicate.

Calculation of Platelet Aggregation in the Presence of Neutrophils or Mononuclear Cells. The addition of leukocytes to the platelet suspension increased the optical density (OD) of the system, so this had to be taken into account when expressing the results. Normally OD = 0% is set with a suspension of washed platelets and OD = 100% is set with Krebs' solution. When other cells were added and the OD increased, this new baseline was taken as 0%. A suspension of the required number of leukocytes in Krebs' solution was used to set the 100% baseline (thrombin does not aggregate leukocytes; see *Results*). The decrease in OD of the washed platelet suspension mixed with leukocytes and stimulated with thrombin was expressed as a percentage of the decrease in OD obtained from the platelet suspension alone following thrombin stimulation. Any platelets contaminating the leukocytes did not contribute to the decrease in OD during thrombin stimulation, for leukocyte suspensions had no effect on aggregation induced by high doses of thrombin. The equation used to express the data is as follows: % maximal decrease in OD = $y/x \cdot 100\%$, where x = % decrease in OD obtainable with thrombin in a suspension of platelets alone, and y = % decrease in OD obtainable with thrombin in the suspension of platelets with neutrophils or platelets with mononuclear cells. The same equation was used to express the data obtained with the U-937 cells.

Coulter Counter Cell Counts. Platelets, neutrophils, and mononuclear cells were counted with a Coulter Counter (model S-Plus IV).

Statistics. Results are expressed as mean ± SEM for n experiments. Student's unpaired t test was used to determine the significance of differences between means, and $P < 0.05$ was taken as statistically significant.

RESULTS

Inhibition by Neutrophils or Mononuclear Cells of Thrombin-Induced Platelet Aggregation. Thrombin (20–80 milliunits/ml) produced a submaximal (70–80% of maximum) and irreversible aggregation of washed platelets within 3 min. When neutrophils (0.5–5 × 10^6) or mononuclear cells (3.5–5 × 10^6) were added to a suspension of washed platelets 1 min before thrombin, the thrombin-induced platelet aggregation was inhibited according to the number of cells added (Fig. 1). U-937 cells (1–6 × 10^6) did not inhibit platelet aggregation (Fig. 1). The inhibition of aggregation induced by neutrophils or mononuclear cells was not due to platelet damage as shown by electron microscopy (results not shown) or to platelet dilution, for addition of corresponding volumes of HHBSS to the platelet suspension did not affect aggregation. Furthermore, the inhibition could not be explained by thrombin uptake by neutrophils or mononuclear cells, for supernatants from suspensions of neutrophils or mononuclear cells

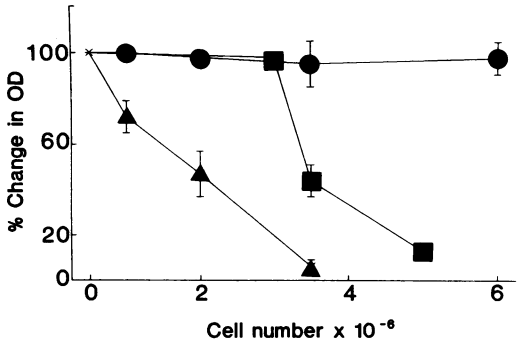

FIG. 1. Effect of different numbers of neutrophils (▲), mononuclear cells (■), or U-937 cells (●) on platelet aggregation induced by thrombin (20–80 milliunits/ml). Neutrophils (1–3.5 × 10^6; n = 6) and mononuclear cells (3–5 × 10^6; n = 5) but not U-937 cells (1–6 × 10^6; n = 3) reduced thrombin-stimulated platelet aggregation. Results are expressed as percent change in optical density (OD). Vertical bars represent SEM of n experiments.

that had been incubated with thrombin produced the same degree of aggregation as that obtained with thrombin alone (n = 4, results not shown). Neutrophils were more potent than mononuclear cells on a cell-number basis as inhibitors of platelet aggregation. The inhibitory activity of neutrophils or mononuclear cells was not seen when a higher concentration of thrombin (400 milliunits/ml) was used (see Fig. 3).

In confirmation of the aggregometer results, measurements in the Coulter Counter showed that the single-platelet count in suspensions of platelets (1–1.5 × 10^8 per ml) alone was significantly ($P < 0.005$, $n = 4$) reduced by thrombin at 20–80 milliunits/ml (Fig. 2). This decrease did not occur when the platelets were previously incubated with neutrophils (5 × 10^6), mononuclear cells (5 × 10^6), or iloprost (100 ng/ml).

The platelet count was significantly increased by incubation of platelets with neutrophils ($P < 0.05$, $n = 4$), mononuclear cells ($P < 0.01$, $n = 4$), or iloprost ($P < 0.01$, $n = 4$) in the absence of thrombin. This was not accounted for by the presence of contaminating platelets in the neutrophil or mononuclear cell fraction (Fig. 2) but was explained by the presence of platelet clumps in the washed platelet suspension. These were detected by the Coulter Counter but were not quantified. The neutrophil or mononuclear cell count was not reduced by thrombin ($n = 2$, results not shown), showing that thrombin did not cause aggregation of leukocytes.

Cell-Free Supernatants Do Not Inhibit Thrombin-Induced Platelet Aggregation. Thrombin-induced platelet aggregation was not inhibited when platelets were incubated with cell-free supernatant from a neutrophil or mononuclear cell suspension ($n = 4$, results not shown). Similarly, the supernatant derived from a suspension of platelets plus neutrophils or platelets plus mononuclear cells had no inhibitory effect (n = 3, results not shown).

Characterization of the Platelet-Inhibitory Factor Derived from Neutrophils or Mononuclear Cells. The inhibition of thrombin-induced platelet aggregation by neutrophils (0.5 × 10^6, Fig. 3*A*) or mononuclear cells (3.5 × 10^6, Fig. 3*B*) was increased in the presence of superoxide dismutase (60 units/ml). The inhibition of platelet aggregation induced by neutrophils or mononuclear cells was not seen when a higher concentration of thrombin was used (400 milliunits/ml, Fig. 3). HbO_2 (10 μM) reversed the inhibition of platelet aggregation induced by neutrophils (from 22 ± 9% to 92 ± 6%, $P < 0.0005$, $n = 10$) and by mononuclear cells (from 22 ± 9% to 89 ± 15%, $P < 0.05$, $n = 6$). Superoxide dismutase (60 units/ml) or HbO_2 (10 μM) did not affect thrombin-induced platelet aggregation at the 70–80% maximal level.

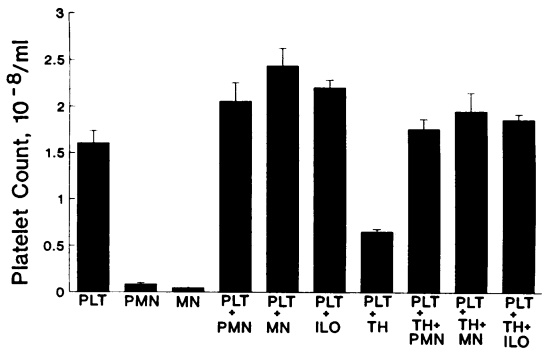

FIG. 2. Effect of neutrophils (polymorphonuclear cells, PMN), mononuclear cells (MN), and iloprost (ILO) on single-platelet count in Coulter Counter experiments. The single-platelet count in suspensions of platelets (PLT) was significantly ($P < 0.005$) reduced by thrombin at 20–80 milliunits/ml (PLT + TH). Incubation of platelets with 5 × 10^6 neutrophils (PLT + PMN) or mononuclear cells (PLT + MN) or with iloprost at 100 ng/ml (PLT + ILO) in the absence of thrombin increased the platelet count ($P < 0.05$, $P < 0.01$, and $P < 0.01$, respectively). The number of platelets contaminating the neutrophil or mononuclear cell fractions are shown (PMN or MN). Addition of thrombin to the platelets incubated with either neutrophils (PLT + TH + PMN), mononuclear cells (PLT + TH + MN) or iloprost (PLT + TH + ILO) did not decrease the platelet count below control (PLT). Mean platelet counts (no. × 10^{-8} per ml) and SEM of four experiments are shown.

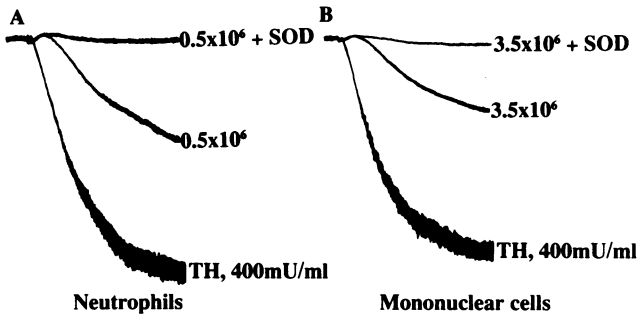

FIG. 3. Effect of superoxide dismutase (SOD, 60 units/ml) on the platelet-inhibitory activity of neutrophils or mononuclear cells. SOD potentiated the inhibition of thrombin (20–80 milliunits/ml)-induced platelet aggregation by neutrophils (0.5 × 10^6) (*A*) or mononuclear cells (3.5 × 10^6) (*B*). The inhibition was completely reversed by a higher concentration of thrombin [TH, 400 milliunits (mU)/ml]. The tracings are representative of 20 experiments.

Neither catalase (1000 units/ml) nor mannitol (1 mM) affected the inhibition of platelet aggregation caused by neutrophils (0.5 – 3.5 × 10^6; $P < 0.4$, $n = 4$) or mononuclear cells (3–4 × 10^6; $P < 0.475$, $n = 6$).

Platelet Cyclic Nucleotides. The inhibition of platelet aggregation induced by neutrophils (4 × 10^6) was accompanied by a significant ($P < 0.0005$, $n = 8$) rise in cGMP levels. No changes in cAMP concentrations were observed (Fig. 4*A*). The inhibition of platelet aggregation induced by mononuclear cells (5 × 10^6) was accompanied by a significant rise in both cGMP ($P < 0.0005$, $n = 8$) and cAMP ($P < 0.05$, $n = 8$; Fig. 4*B*).

Inhibition of Arginine Metabolism. Preincubation of neutrophils (2–3.5 × 10^6) or mononuclear cells (3–5 × 10^6) for 50 min with N^G-monomethyl-L-arginine methyl ester (30–300 μM) reduced their ability to inhibit thrombin (20–40 milliunits/ml)-stimulated platelet aggregation. L-Arginine (100 μM), but not D-arginine (100 μM), counteracted the effect of N^G-monomethyl-L-arginine methyl ester. Tracings representative of four experiments are shown in Fig. 5.

DISCUSSION

Human neutrophils and mononuclear cells release a factor that inhibits thrombin-induced platelet aggregation. Although this factor has not been conclusively identified, it shows a pharmacological profile similar to that of EDRF (NO). One characteristic of EDRF and exogenous NO is that they survive longer when Krebs' buffer contains superoxide dismutase (28, 29). Thus, superoxide dismutase potentiated the inhibitory activity of this NO-like factor, but this effect might be due to inactivation of superoxide (O_2^-) only, for these anions alone potentiate platelet aggregation (4). HbO_2 oxidizes NO to NO_3^-, thus destroying its biological activity (30, 31). HBO_2 reversed the inhibitory activity of the NO-like factor, demonstrating that the biological activity of this factor, like that of NO, is lost in the presence of HBO_2.

The inhibition of platelet aggregation by NO occurs through the activation of guanylate cyclase (32, 33). Similarly, platelet inhibition by the NO-like factor from neutrophils was accompanied by an increase in cGMP but not in cAMP. This is further evidence that the factor is NO. Interestingly, the inhibition of platelet aggregation by mononuclear cells was accompanied by increases in both cGMP and cAMP. Although mononuclear cells and neutrophils can

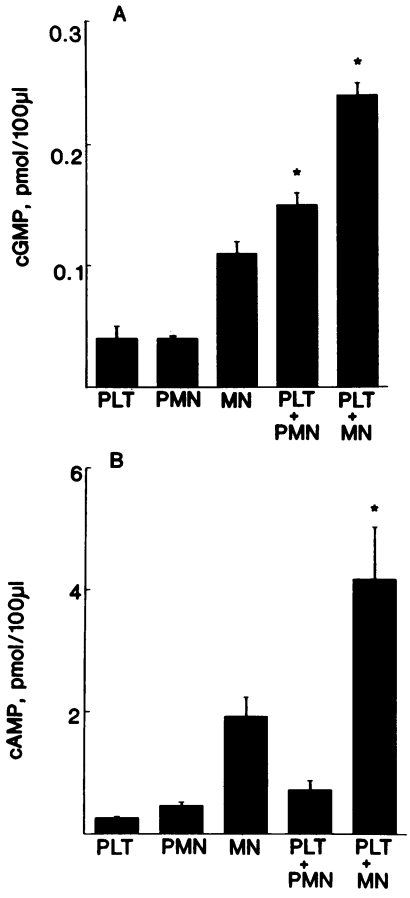

FIG. 4. Concentrations of cyclic nucleotides in platelets (PLT), neutrophils (PMN), or mononuclear cells (MN). Measurements were carried out in the presence of 2 μM 3-isobutyl-1-methylxanthine. (*A*) Mixing of neutrophils (4×10^6) or mononuclear cells (5×10^6) with platelets (PLT + PMN or PLT + NM) significantly raised the levels of cGMP above those in platelets and neutrophils or platelets and monocytes added together (asterisk; $P < 0.0005$, $n = 8$). (*B*) The addition of neutrophils (4×10^6) to the platelets (PLT + PMN) did not increase cAMP ($P < 0.3$, $n = 8$), whereas the addition of mononuclear cells to the platelets (PLT + MN) significantly ($P < 0.05$, $n = 8$) increased cAMP when compared to the levels of cAMP if the effect were an additive one (PLT and MN).

release prostaglandins that activate adenylate cyclase, a cyclooxygenase product was excluded by treating the cells with indomethacin. Whether lipoxygenase products or other fatty acids released by the mononuclear cells are involved in the stimulation of adenylate cyclase remains to be studied.

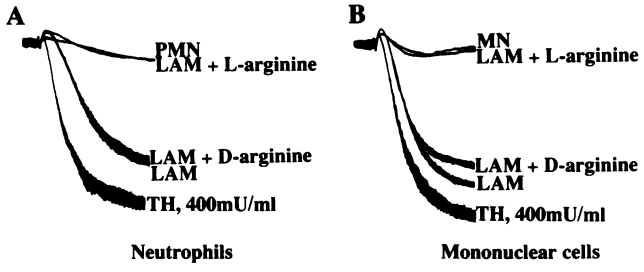

FIG. 5. Effect of N^G-monomethyl-L-arginine methyl ester (LAM) on the inhibition of thrombin (40 milliunits/ml)-induced platelet aggregation by neutrophils (PMN, 3×10^6) or mononuclear cells (MN, 4×10^6). Preincubation of PMN (*A*) or MN (*B*) for 50 min with LAM (100 μM) reversed their platelet-inhibitory activity. L-Arginine (100 μM) but not D-arginine (100 μM) counteracted the effect of LAM. The inhibitory activity of PMN or MN cells was completely reversed by a higher concentration of thrombin [TH, 400 milliunits (mU)/ml]. Tracings are representative of four similar experiments.

L-Arginine is the precursor for NO_2^- and NO_3^- synthesis in macrophages (18, 19) and of NO synthesis in macrophages and endothelial cells (15, 21, 34, 35). The L-arginine analogue N^G-monomethyl-L-arginine inhibits arginine metabolism and thus inhibits NO or NO_2^- and NO_3^- release from macrophages (21, 34) and endothelial cells (35, 36). N^G-Monomethyl-L-arginine methyl ester is as potent an inhibitor of EDRF release as is N^G-monomethyl-L-arginine (J.R.V., G.dN., and J. Mitchell, unpublished data) and prevented the release of the NO-like factor. As in the endothelial cells with N^G-monomethyl-L-arginine, this was overcome by L-arginine but not by D-arginine. These results strongly suggest that the platelet-inhibitory factor is NO and that, as in endothelial cells, its precursor is L-arginine.

Activated leukocytes release H_2O_2 and OH·, the former reported by some to inhibit platelet aggregation (5, 6, 37). Since neither catalase (H_2O_2 scavenger) nor mannitol (OH· scavenger) affected the activity of the inhibitory factor, we can conclude that these radicals do not contribute to the breakdown of this factor, nor are they responsible for the observed platelet-inhibitory effect.

The observations from the aggregometer experiments were extended by the use of a Coulter Counter. The inhibition of thrombin-induced platelet aggregation was dependent on the number of nucleated cells. In the absence of thrombin, the addition of nucleated cells or of iloprost increased the platelet count. This must have been due to disaggregation of the small platelet clumps detected by the Coulter Counter. Since thrombin did not decrease the single-leukocyte count, it could not have induced either neutrophil or mononuclear cell aggregation.

The inhibition of platelet aggregation induced by leukocytes was not due to a nonspecific particle effect, since U-937 human monocytic cells did not inhibit platelet aggregation. Further, the inhibitory activity of the leukocytes was reversed by thrombin, HBO_2, and N^G-monomethyl-L-arginine methyl ester.

Thus, we have shown that human neutrophils and mononuclear cells release a platelet-inhibitory factor that has a pharmacological profile similar to that of EDRF (NO). It has been shown that nonstimulated human neutrophils do not release EDRF as evidenced by experiments using rat aortic rings (38). Therefore, another conclusion to be drawn from these results is that the enzyme responsible for the release of the NO-like factor can be activated by mild stimulation such as stirring that takes place during aggregation experiments. The release of NO-like activity from rat neutrophils (8, 39), or murine macrophages (21) had to be elicited by the use of strong stimuli such as oyster glycogen or *Escherichia coli* lipopolysaccharide. If NO is indeed released when contact occurs between platelets and leukocytes, such a mechanism could have implications in limiting the onset and propagation of thrombogenic processes *in vivo* in which the participation of extracellular release of EDRF (NO) would be limited by its destruction by superoxide anions and haptoglobin–hemoglobin complexes in plasma (28, 40). It may be that if leukocytes can influence the course of hemostasis and thrombosis by modulating platelet function, an approach to therapy of hemorrhagic and thrombotic diseases could be opened. Our experiments suggest that the U-937 monocytic cells do not make NO or that they require activation with stronger or different stimulants.

NO has other properties besides its vasorelaxant and platelet-inhibitory activity. It inhibits neutrophil aggregation (41), increases vascular permeability (42), inhibits mitogen release from platelets (43), and has a cytoprotective effect on platelets (44). Both neutrophils and mononuclear cells are inflammatory cells armed with a potent capacity to undergo

the oxygen respiratory burst (45, 46) and in doing so to release superoxide anions into the extracellular medium (47, 48). In the light of these observations it is possible that the ability of these cells both to release NO and to inactivate it by the generation of superoxide anions could represent an important regulatory mechanism in the inflammatory response. Further evidence that human neutrophils release NO has been recently obtained by chemiluminescence studies (49, 50).

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