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Platelet-leukocyte interaction: activation of rabbit platelets by FMLP-stimulated neutrophils

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1 The effect of the chemotactic peptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) was studied on cells in whole rabbit blood or on a mixture of purified rabbit platelets and neutrophils.

2 In blood, FMLP triggered cell aggregation (measured by electrical impedance) which was dependent upon the concentration of FMLP (9.9 ± 0.7 and 5.2 ± 1.2 ohms at 1 and 0.01 μM FMLP respectively). This aggregation was accompanied by a strong decrease in platelet counts (54.6 ± 6.0 and 45.6 ± 3.8% for 1 and 0.01 μM FMLP respectively) and by a smaller decrease in neutrophil counts (25.0 ± 1.9 and 12.9 ± 1.7% at 1 and 0.01 μM FMLP respectively).

3 When purified platelets and neutrophils were co-incubated, the addition of 0.1 μM induced a marked aggregation (50.0 ± 1.6 vs. 19.5 ± 1.6% of light transmission, $n = 8$, $P < 0.001$), ATP secretion (8.4 ± 1.0 vs. 0.1 ± 0.1 nmol ml^{-1}, $n = 6$, $P < 0.001$) and a decrease in platelet counts. FMLP induced aggregation of purified neutrophils and release of lysozyme but lacked direct platelet-stimulating effects. The release of lactate dehydrogenase, a cytoplasmic marker and lysozyme were unchanged under the interaction conditions.

4 Platelet activation was reduced by about 30% with 100 μM aspirin or indomethacin and by about 70% with 100 μM BW 755C. Two Paf-acether antagonists, BN 52021 (100 μM) and WEB 2086 (1 μM) suppressed platelet activation by 70–80%.

5 The supernatant of FMLP-stimulated neutrophils induced platelet activation only when bovine serum albumin was present. Rabbit neutrophils stimulated in the presence of serum albumin by 1 μM FMLP formed 2 nM Paf-acether of which half was released to the extracellular medium.

6 Our results indicate that the stimulation of neutrophils by FMLP induces platelet activation in whole blood and on isolated cells and that both arachidonic acid-metabolites and Paf-acether participate in platelet activation.

Introduction

Arterial thrombi are composed primarily of aggregated platelets, but are also rich in granulocytes (Henry, 1965; Bourgain *et al.*, 1985). Platelets and leukocytes are in fact associated and interact under different conditions and this interaction may influence the course of thrombosis and/or inflammation. Several mechanisms of leukocyte-platelet interaction have been described to explain the platelet-dependent increase of vascular permeability and inflammatory lesions and different pathways for cooperation between platelets and neutrophils to synthesize mediators of inflammation are known. Thus platelet factor 4, a polypeptide stored in the granules of human platelets, stimulates human basophils to release histamine (Brindley *et al.*, 1983). Arachidonic acid released from platelets can be converted by neutrophils to leukotriene B_4, a potent chemotactic and leukocyte marginating agent (Kuehl *et al.*, 1984; Marcus *et al.*, 1982). On the other hand, 12-hydroxyeicosatetraenoic acid (12-HETE) from stimulated platelets is transformed by resting neutrophils into 5,12-diHETE (Maclouf *et al.*, 1982) and 12,20-diHETE (Marcus *et al.*, 1984, 1987). Recently, we described a novel platelet-neutrophil cooperation in which lyso compound (deacetylated Paf-acether) released from activated platelets is acetylated by neutrophils, resulting in a marked increase of Paf-acether production (Coëffier *et al.*, 1984; Benveniste & Coëffier, 1984 and unpublished observations).

The tripeptide N-formyl-L-methionyl-L-leucyl-L-

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phenylalanine (FMLP) is a potent polymorphonuclear leukocyte secretagogue and chemotactic agent (Painter *et al.*, 1984). Its intravenous administration to the guinea-pig induces bronchoconstriction and dose-dependent leukopenia accompanied by mild thrombocytopenia (Boukili *et al.*, 1985; Berend *et al.*, 1985). In the rabbit, the intravenous infusion of FMLP triggers neutropenia and additional thrombocytopenia, which is prevented by neutrophil depletion (Issekutz *et al.*, 1983). Similarly, the intravenous injection of complement factors, known to activate neutrophils *in vitro*, results in the emergence of platelet and neutrophil aggregates in the guinea-pig pulmonary vessels and heart (Stimler *et al.*, 1980).

The purpose of our study was to determine the mechanism of platelet and neutrophil interaction, to answer the question whether granulocytes can influence platelet function *in vitro* and, if so, which factor(s) account for this interaction and which drugs can modulate it. Our results indicate that FMLP-stimulated neutrophils induce platelet activation (aggregation, secretion) in whole rabbit blood and in platelet-neutrophil co-incubates, which is largely mediated by formation of arachidonic acid metabolites and Paf-acether.

Methods

Whole blood aggregation studies

Blood was collected from the central ear artery of adult New Zealand white rabbits of either sex in Falcon tubes containing 3.8% (w/v) sodium citrate (1 vol/9 vol of blood) and heparin ($5\,iu\,ml^{-1}$). Blood (1 vol) was diluted with 3 vol of buffer of the following composition (mM): NaCl 137.0, KCl 2.6, $NaHCO_3$ 0.6, NaH_2PO_4 0.02, glucose 5.6, $MgCl_2$ 2.0, $CaCl_2$ 1.3, pH 7.2 by a modification of the technique of Russell-Smith *et al.* (1981). In these conditions the number of leucocytes and platelets in the reaction medium was respectively of $1.8 \pm 0.1 \times 10^6$ per ml and $1.0 \pm 0.1 \times 10^8$ per ml (mean ± s.e.mean, $n = 24$).

Platelet aggregation was monitored with a whole blood aggregometer (Chronolog) at 900 r.p.m. and at 37°C in 1 ml siliconized glass cuvettes. In this technique, two electrodes are inserted into the blood sample or cell suspension (Russell-Smith *et al.*, 1981). During the initial contact with the solution, a monolayer of cells deposits onto the electrodes. After waiting 3 min to obtain a baseline, aggregating agents were added to the cuvette through the needle guide. The suspended cells aggregate onto this monolayer and thus coat the electrodes, and this is detected by the fall in conductance between the two electrodes. The extent of aggregation is calibrated in terms of changes in electrical impedance (ohm) and measured 5 and 10 min after addition of the agonists. At these two times, aliquots were collected for counting neutrophils and platelets with a Coulter counter ZBI (Coultronics).

Preparation of rabbit polymorphonuclear neutrophils

Blood was collected from the central ear artery of rabbits in Falcon tubes containing ACD (trisodium citrate 66 mM, citric acid 85 mM and glucose 111 mM; 1 vol ACD for 6 vol of blood). After sedimentation for 60 min with gelatin 2.5% (1 vol for 9 vol of blood), two volumes of the upper leukocyte suspension was recovered and layered on one volume of Ficoll-Paque. After centrifugation (400 *g*, 20 min, 20°C), the bottom layer was resuspended in a 4.2 mM HEPES-buffered solution containing (mM): NaCl 137.0, KCl 2.6, glucose 5.6, pH 7.4. The leukocyte preparation was submitted to an osmotic shock in order to eliminate contaminant erythrocytes: 1 volume of leukocyte suspension with 3 volumes of distilled water were incubated for 40 s, then 1 volume of 0.58 M NaCl was added for 15 s followed by addition of 40 volumes of HEPES-buffer. After 2 washes by centrifugation (350 *g*, 10 min, 20°C) and resuspension in HEPES-buffer, the cells were counted and resuspended in the washing buffer in order to adjust the concentration to 10×10^6 neutrophils per ml. The suspension was supplemented with $MgCl_2$ 1.3 mM and $CaCl_2$ 1.0 mM just before stimulation.

Preparation of washed rabbit platelets

Blood was collected as for neutrophils and platelets were prepared as described by Ardlie *et al.* (1970). Platelet-rich plasma obtained by centrifugation of blood (375 *g*, 20 min; 20°C) was centrifuged (1400 *g*, 15 min; 20°C) and the platelet pellet was gently resuspended in a Tyrode buffer (mM): NaCl 137.0; KCl 2.6; $NaHCO_3$ 11.9; NaH_2PO_4 0.42, $MgCl_2$ 2.0, glucose 5.6; albumin 0.35%; pH 6.5; supplemented with prostacyclin 10 nM and EGTA 2 mM. After centrifugation (1400 *g*; 10 min; 20°C) the supernatant was removed and the platelet pellet was resuspended in the same buffer supplemented with prostacyclin 10 nM. After a last wash, the platelet pellet was resuspended in Tyrode buffer supplemented with $CaCl_2$ 1.8 mM and in which the concentration of $MgCl_2$ was reduced to 1.0 mM, pH 7.35 (Tyrode -Ca^{2+}-Mg^{2+}).The platelets were counted in order to adjust the concentration to 1×10^9 platelets per ml. No polymorphonuclear leukocytes were detect upon examination of the platelet preparations by optical microscopy. The final platelet suspension was kept 15 min at room temperature before use.

Aggregation studies with isolated neutrophils and platelets

Neutrophils (10×10^6 per ml) in HEPES-buffer supplemented with $CaCl_2$ 1.3 mM and $MgCl_2$ 1.0 mM and platelets (10×10^8 per ml) in Tyrode -Ca^{2+}-Mg^{2+} were suspended either alone or in combination in order to obtain a final concentration of 5×10^6 neutrophils per ml and 5×10^8 platelets per ml. Aggregation was followed by two methods: (1) by the difference of light transmission measured in a Chronolog aggregometer (0.4 ml reactional mixture) with stirring (1100 r.p.m.) and at 37°C; (2) by the change in electrical impedance measured with the whole blood aggregometer at 900 r.p.m. and at 37°C in 3 ml siliconized glass cuvettes (1 ml reactional mixture). In this case, neutrophils and platelets were counted 5 and 10 min after the addition of the agonist. In both conditions, a 20 µl aliquot was collected 0.5 min before and 1.5 and 3 min after the addition of the aggregating agent, for monitoring the platelet release reaction according to the concentration of ATP released to the extracellular medium (luciferine-luciferase technique), as described by Holmsen & Weiss (1972).

Assays of lysozyme, β-N-acetyl-glucosaminidase (NAGA) and lactate dehydrogenase (LDH)

Neutrophils (5×10^6 per ml) in HEPES-buffer supplemented with $CaCl_2$ 1.3 mM and $MgCl_2$ 1.0 mM and platelets (5×10^8 per ml) in Tyrode -Ca^{2+}-Mg^{2+} were suspended either alone or in combination during 30 min at 37°C without agonist and in the presence of different concentrations of FMLP. The reaction was stopped by the addition of EDTA (5 mM, final concentration) and centrifugation (1100 g; 15 min; 4°C). Supernatants and pellets were stored separately at −20°C for lysozyme and NAGA and at +4°C for LDH determinations. We measured the release of lysozyme, a neutrophil specific and azurophil granules enzyme (Litwack, 1955) and of β-N-acetyl-glucosaminidase (NAGA, E.C. 3.2.1.30), a marker for the azurophil granules of neutrophils (Falloon & Gallin, 1986) and for the α granules of platelets (Gordon & Milner, 1976). Platelet and neutrophil viability was checked by measuring the release of LDH, a cytoplasmic enzyme (Wroblewski & Ladue, 1955). Enzyme release is expressed as the percentage of the total enzyme content measured after lysing the cells with 0.1% Triton × 100.

Bioassay for Paf-acether formation

Paf-acether formed by neutrophils following stimulation with FMLP was evaluated on rabbit washed platelets, neutrophils (5×10^6 per ml) being stimulated for 3 min with FMLP 1 µM with or without lipid-free bovine serum albumin (BSA, 0.25%, w/v) and immediately centrifuged in eppendorf tubes. The supernatants and the pellets suspended in HEPES-buffer were extracted by the method of Bligh & Dyer (1959), dried with nitrogen at 40°C, the residue being suspended in 0.15 M NaCl containing 0.25% BSA (pH 7.4). Aggregations were performed at 37°C with stirring, using platelets pretreated with aspirin (100 µM) for 15 min in order to suppress cyclo-oxygenase activity. The Tyrode -Ca^{2+}-Mg^{2+} was furthermore supplemented with the CP/CPK complex (1 mM and 10 u ml^{-1} respectively) in order to prevent aggregation by ADP. A calibration curve was established with synthetic Paf-acether, the threshold for a minimal response being 0.08 nM of Paf-acether.

Electron-microscopy studies

Washed rabbit platelets (5×10^8 per ml) were preincubated for 1 min at 37°C while stirring (1100 r.p.m.) and then stimulated with FMLP 1 µM or Paf-acether 10 nM. Purified neutrophils (5×10^6 per ml) and platelets and neutrophils, were preincubated and stimulated in the same conditions. Three minutes after the addition of the agonist, the samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h and centrifuged at low speed. Pellets were rinsed in 0.1 M cacodylate buffer and post-fixed for 1 h with 1% osmium tetroxide in the same buffer. The specimens were dehydrated in ethanol series (25–100%) and embedded in Epon 812. Ultrathin sections were stained conventionally with uranyl acetate and lead citrate (Reynolds, 1963).

Materials

Lipid-free bovine serum albumin (BSA); N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP); N-tert-butyloxy-carbonyl-L-methionyl-L-leucyl-L-phenylalanine (Boc-MLP); indomethacin; arachidonic acid (AA); adenosine 5′-triphosphate (ATP); creatine phosphate (CP); creatine phosphokinase (CPK); N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES); superoxide dismutase, catalase and Triton X 100 were obtained from Sigma Chemical Co, St-Louis, MO, USA. Ficoll-Paque was from Pharmacia, Uppsala, Sweden; aspirin lysine salt (Aspegic) from Laboratoires Egic, Amilly, France; ethylene-glycol-tetra-acetate (EGTA), tris (hydroxymethyl) amino-methane (Tris) and gelatin of microbiology grade were from Merck, Darmstadt, GFR; heparin from Laboratoires Choay, Paris, France; synthetic Paf-acether (1-O-hexadecyl-2-acetyl-*sn*-glycerly-3-phosphorylcholine) from Bachem, Bubendorf, Switzerland. The luciferine-luciferase enzymatic preparation (Lumit PM) was obtained from Lumac (Schaes-

berg, The Netherlands) and used as recommended for the determination of ATP released from platelets, using a Pico-ATP automatic device (Jobin-Yvon, France). Collagen was purchased from Horm-Chemie (Munchen, RFA). BN 52021 (9-H-1,7a- (epoxymethano)-1H,6aH-cyclopenta-(c)-furo-(2,3b)-(3′,2′; 3,4) cyclopenta-(1,2d)-furan 5,9,12(4H)-trione, 3-tert-butyl- hexahydro-4,-7b-11- hydroxy-8-methyl) supplied by IHB-IPSEN (Le Plessis-Robinson, France) was solubilized at 1 mg ml^{-1} with NaOH 0.1N; the pH was immediately adjusted to 7.4 with HCl 1N and further diluted in saline. WEB 2086 (3-(4- (2-chlorophenyl)- 9-methyl-6H-thieno (3,2-f) (1,2,4) triazolo (4,3-a) (1,4)-diazepine-2-yl) -1-(4-morpholinyl)-1-propanone) was from Boehringer Ingelheim KG, FRG. Nafazatrom (Bay g 6575; 1-((2-β-naphtyloxy) ethyl)-3-methyl-2-pyrazolin-5-one) was dissolved in 0.1 N NaOH and diluted in buffer to final concentrations. BW 755C ((3-amino-1-(*m*-(trifluoromethyl) phenyl)-2-pyrazoline) and prostacyclin were gifts from Dr S Moncada (The Wellcome Research Laboratories, Beckenham, U.K.).

Results

Effect of FMLP in whole rabbit blood

The addition of FMLP to whole rabbit blood was followed by a concentration-dependent increase in electrical impedance after a time lag of about 2 min. In the same conditions, collagen (1 μg ml^{-1}) and Paf-acether (0.3 nM) induced an increase of impedance equivalent to that due to 1 μM FMLP (Figure 1, Table 1). Platelet aggregation was accompanied by a marked decrease in platelet counts (55% for 1 μM FMLP) and by a smaller decrease in leukocyte counts (25% for 1 μM FMLP), the basal number of platelets and leukocytes in whole rabbit blood being respectively of $1.0 \pm 0.1 \times 10^8$ per ml (mean ± s.e.mean, $n = 21$) and $1.8 \pm 0.1 \times 10^6$ per ml (mean ± s.e.mean, $n = 24$) (Table 1).

Interference of potential inhibitors with the effect of FMLP in whole rabbit blood.

The FMLP-induced aggregation of platelets and of neutrophils in whole rabbit blood was inhibited by about 50% by the chemotactic peptide antagonist (Spisani *et al.*, 1986) Boc-MLP (1 μM), which was inactive against collagen-induced aggregation (Table 2). Aspirin (100 μM) and the lipoxygenase inhibitor (Honn & Dunn, 1982) Nafazatrom (100 μM) inhibited slightly the increase of impedance induced by FMLP, the decrease in leukocyte and platelet counts induced by FMLP being unaffected by both drugs. The two Paf-acether antagonists, BN 52021 (100 μM) (Braquet *et al.*, 1985) and WEB 2086 (1 μM) (Casals-Stenzel *et al.*, 1986) suppressed totally the increase of impedance and the decrease in leukocytes and in platelets counts triggered by 1 μM FMLP and by 0.03 nM Paf-acether. At the same concentrations, BN 52021 and WEB 2086 were less effective against activation induced by a higher concentration of Paf-acether (30 nM) and against collagen (1 μg ml^{-1}).

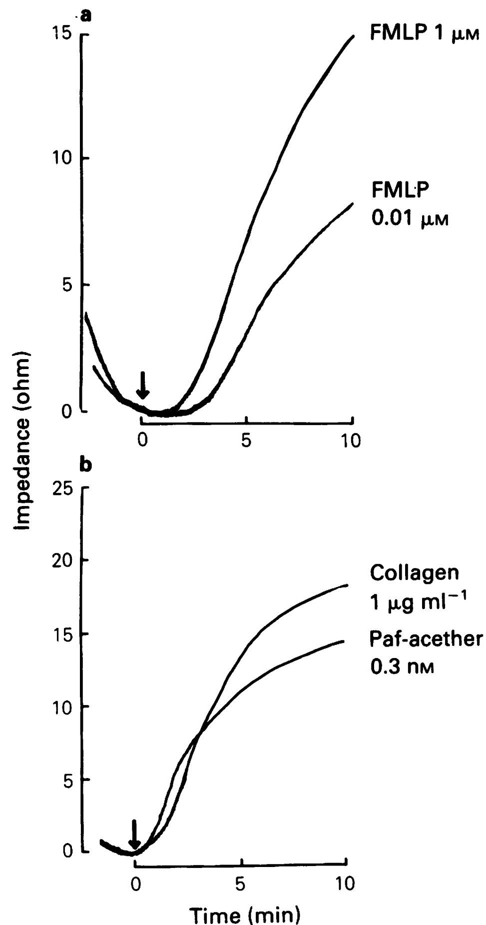

Figure 1 Aggregation induced by FMLP (a), collagen and Paf-acether (b) in whole rabbit blood. Rabbit blood was diluted four fold with buffer as described in 'Methods' and platelet aggregation was monitored on 1 ml samples with the whole blood aggregometer at 900 r.p.m. and 37°C. After 3 min to obtain a stable baseline, aggregating agents were added at volumes below 10 μl. Aggregation of cells was detected by the reduction in conductance between the electrodes and was calibrated as changes in electrical impedance (ohm). The curve is representative of 17–25 experiments.

Table 1 Effects of FMLP, Paf-acether and collagen in whole rabbit blood

Stimulating agent and concentration		*Intensity of aggregation* (impedance in ohms)	*Decrease of cell counts (%)* Neutrophils	Platelets	n
0		1.5 ± 0.4	0.7 ± 0.3	21.2 ± 4.1	19
FMLP	1 μM	9.9 ± 0.7	25.0 ± 1.9	54.6 ± 6.0	42
FMLP	0.01 μM	5.2 ± 1.2	12.9 ± 1.7	45.6 ± 3.8	25
Collagen	1 μg ml^{-1}	19.6 ± 1.1	53.6 ± 3.6	71.6 ± 5.6	28
Paf-acether	30 nM	20.4 ± 1.3	65.7 ± 0.7	85.3 ± 0.3	14
Paf-acether	0.3 nM	13.7 ± 1.3	21.1 ± 8.3	74.9 ± 4.9	17

Aggregation was monitored with a whole blood aggregometer at 900 r.p.m. and at 37°C. Two electrodes were inserted in the four fold diluted blood sample. After 3 min, the aggregating agents were added. Aggregation was calibrated in terms of changes in electrical impedance in ohms. Five min after the addition of the agonists, aliquots were collected for counting platelets and neutrophils. The decrease in cell counts is expressed as a percentage. The results are the mean ± s.e.mean of the number of indicated experiments (n). All the results are significant ($P < 0.001$) as compared to controls in the absence of agonist.

Table 2 Effect of inhibitors in whole rabbit blood

Agonist and concentration	*Inhibitor*	(μM)	*% inhibition* *Impedance*	*Decrease of cell counts of* *Neutrophils*	*Platelets*	n
FMLP	Boc-MLP	1	48.4 ± 22.5	47.0 ± 23.4	26.2 ± 10.3*	7
1 μM	Aspirin	100	38.3 ± 36.5	2.0 ± 3	8.3 ± 8.2	5
	Nafazatrom	100	20.7 ± 14.5	0	6.4 ± 9.4	9
	BN 52021	100	99.2 ± 14.2*	91.6 ± 40.8	84.9 ± 13.2*	3
	WEB 2086	1	79.3 ± 11.9*	98.0 ± 18.2*	61.1 ± 14.3*	3
FMLP	Boc-MLP	1	46.6 ± 17*	64.0 ± 33.7	7.0 ± 10.9	7
0.01 μM	Aspirin	100	0	27.6 ± 50	11.9 ± 10.2	5
	Nafazatrom	100	74.4 ± 25.9*	16.9 ± 24	7.1 ± 13.0	6
Collagen	Boc-MLP	1	0	15.4 ± 18.0	0	3
1 μg ml^{-1}	Aspirin	100	34.7 ± 11.5*	48.2 ± 15.4*	18.11 ± 5.5*	7
	Nafazatrom	100	6.1 ± 13.5	8.0 ± 3.5	5.5 ± 6.7	5
	BN 52021	100	4.1 ± 18.5	23.2 ± 25.9	6.7 ± 6.4	4
	WEB 2086	1	23.1 ± 9.8	22.3 ± 36.9	7.8 ± 6.2	4
Paf-acether	BN 52021	100	42.1 ± 12.1*	52.9 ± 8.8**	8.6 ± 4.2	5
30 nM	WEB 2086	1	68.2 ± 18.9*	84.2 ± 8.3***	37.4 ± 18.9	5
Paf-acether	BN 52021	100	99.1 ± 28.7*	69.4 ± 52.6	79.0 ± 8.3**	5
0.03 nM	WEB 2086	1	99.2 ± 25.3*	78.3 ± 61.4	67.8 ± 4.5***	5

Aggregation and cell counts were studied as in Table 1. Blood was preincubated at 37°C for 2 min with Boc-MLP and Nafazatrom, for 1 min with BN 52021 and WEB 2086 or at room temperature for 15 min with aspirin. The results are expressed as percentage inhibition (mean ± s.e.mean of the number of indicated experiments (n). (*$P < 0.01$; **$P < 0.05$; ***$P < 0.001$; t test on unpaired variables).

Effect of FMLP on the mixture of isolated platelets and neutrophils

FMLP induces aggregation of rabbit neutrophils but lacks direct platelet-stimulating effects. Indeed the addition of FLMP to washed platelets alone induced neither aggregation nor a decrease in platelet counts (Figure 2). By contrast, when washed rabbit platelets were incubated in the presence of isolated neutrophils, the addition of 1 μM FMLP induced platelet aggregation and ATP secretion, two parameters of platelet activation (Figure 2). The stimulation of neutrophils induced by FMLP was concentration-dependent (Figure 3) with a maximal effect at 0.1 μM FMLP.

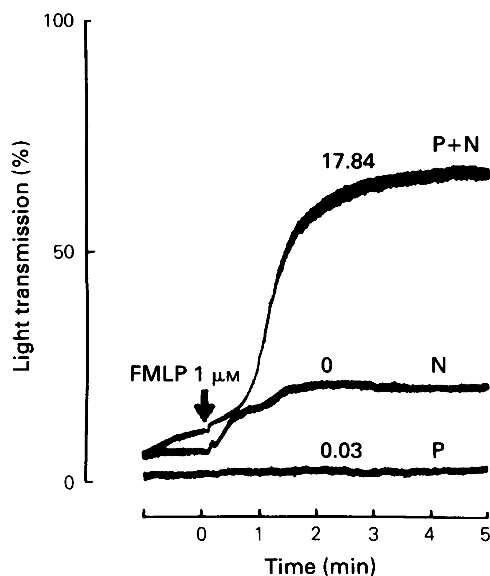

Figure 2 Activation of rabbit platelets by FMLP-stimulated neutrophils. Washed rabbit platelets (P) (0.2 ml; 10 × 10⁸ per ml) were preincubated for 1 min at 37°C with stirring (1100 r.p.m.) and then stimulated by FMLP (1 μM). Purified neutrophils (N) (0.2 ml; 10 × 10⁶ per ml) and the co-incubate of platelets and neutrophils (P + N) were stimulated under the same conditions. Three minutes later a 20 μl aliquot was collected for ATP determination. The figures above the curves indicate the amounts of ATP secreted (nM). The results are representative of 8 experiments.

Likewise, the increase of aggregation when washed rabbit platelets and purified neutrophils were incubated together was also dependent upon FMLP concentration, with a maximal and very significant effect at 0.1 μM ($P < 0.001$) (Figure 3). During platelet-neutrophil interaction, aggregation triggered by FMLP started after a time lag of about 60 s, at 90 s the mild aggregation elicited by FMLP was concentration-dependent and became maximal at 3 min (Figure 4). Aggregation of rabbit platelets in the presence of

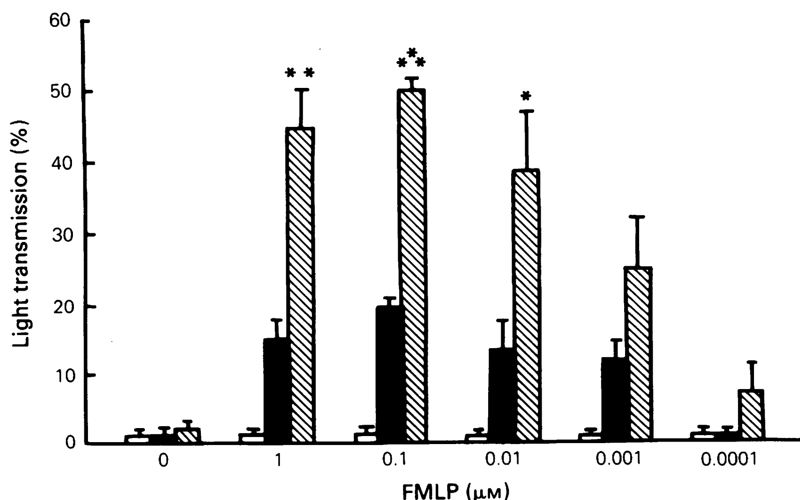

Figure 3 Aggregation of rabbit platelets in the presence of FMLP-stimulated neutrophils. Washed rabbit platelets (open columns) (0.2 ml; 10 × 10⁸ per ml) were preincubated for 1 min at 37°C with stirring (1100 r.p.m.) and stimulated by different concentrations of FMLP. Purified neutrophils (solid columns) (0.2 ml; 10 × 10⁶ per ml), and platelets and neutrophils (hatched columns), were preincubated and stimulated under the same conditions. Aggregation in percentage of light transmission was measured 3 min after the addition of the stimulating agent. The results are the mean of 8 experiments with s.e.mean shown by vertical lines. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, t test on the results from unpaired samples of FMLP-treated neutrophils and platelets, and FMLP-treated neutrophils.

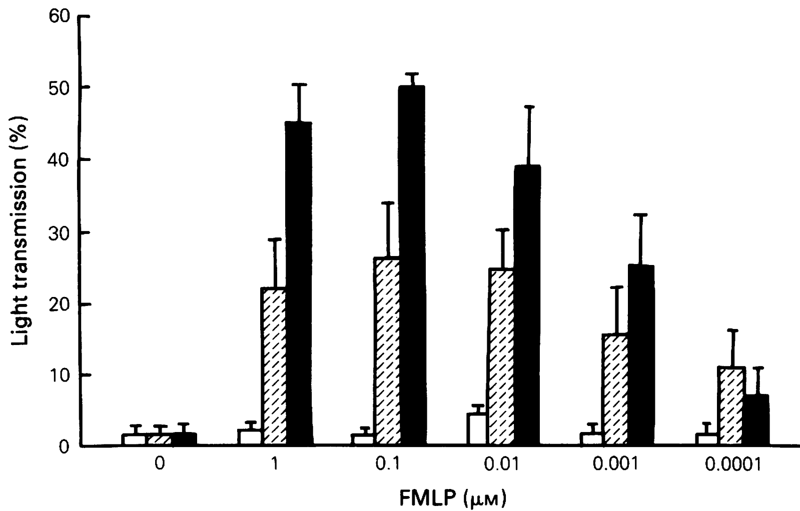

Figure 4 Kinetics of platelet aggregation in the presence of FMLP-stimulated neutrophils. Washed rabbit platelets (0.2 ml; 10×10^8 per ml) and purified neutrophils (0.2 ml; 10×10^6 per ml) were co-incubated for 1 min at 37°C with stirring (1100 r.p.m.) and then stimulated by different concentrations of FMLP. Aggregation as percentage of light transmission was measured 0.5 min (open columns), 1.5 min (hatched columns) and 3 min (solid columns) after addition of the stimulating agent. The results are the mean of 8 experiments with s.e.mean shown by vertical lines.

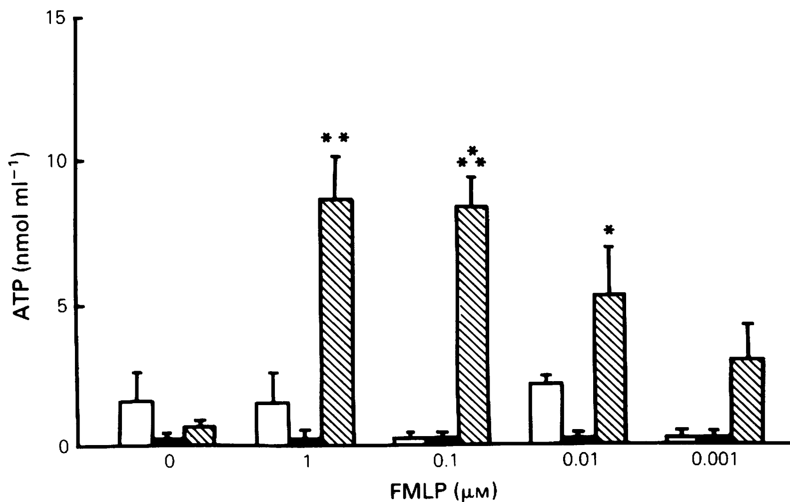

Figure 5 Secretion of ATP from rabbit platelets in the presence of FMLP-stimulated neutrophils. Washed rabbit platelets (open columns) (0.2 ml; 10×10^8 per ml) were preincubated for 1 min at 37°C with stirring (1100 r.p.m.) and then stimulated by FMLP. Purified neutrophils (solid columns) (0.2 ml; 10×10^6 per ml), and platelets and neutrophils (hatched columns) were co-incubated and stimulated in the same conditions. Three min after the addition of the stimulating agent, a 20 μl aliquot was collected for ATP determination. The results in nmol ATP ml^{-1} are the mean of 6–12 experiments; vertical lines show s.e.mean. $*P<0.05$; $**P<0.01$; $***P<0.001$, t test on the results from unpaired samples of FMLP-treated neutrophils and platelets, and FMLP-treated platelets.

Table 3 Decrease of platelet and neutrophil counts (%) after stimulation by FMLP for 5 min

Experimental conditions	Cell type counted	FMLP concentration (μM) 0	1	0.01	0.001	0.0001
Neutrophils alone	Neutrophils	14.6 ± 4.9 (9)	43.8 ± 5.0 (5)**	46.5 ± 10.3 (4)**	18.3 ± 13.6 (3)	0 (3)
Platelets alone	Platelets	2.9 ± 2.5 (12)	5.3 ± 2.5 (5)	ND	ND	ND
Neutrophils +	Neutrophils	10.6 ± 4.0 (8)	56.3 ± 10.3 (6)***	40.7 ± 14.1 (4)*	33.2 ± 9.9 (5)*	23.0 ± 13.4 (5)
Platelets	Platelets	4.1 ± 2.5 (8)	68.0 ± 14.9 (6)***	83.0 ± 10.0 (4)***	78.8 ± 9.3 (5)***	23.2 ± 17.8 (5)

Washed rabbit platelets (1 ml; 5 × 10⁸ per ml) were preincubated for 1 min at 37°C in the whole blood aggregometer as indicated in Table 1 and were stimulated by FMLP. Purified neutrophils (1 ml; 5 × 10⁶ per ml), and platelets (0.5 ml; 10 × 10⁸ per ml) and neutrophils (0.5 ml; 10 × 10⁶ per ml) were preincubated and stimulated with FMLP under the same conditions. Five min after adding the agonists, aliquots were collected for counting platelets and neutrophils. The results are expressed as percentage decrease (mean ± s.e.mean of number of experiments indicated in parentheses): $*P<0.01$; $**P<0.05$; $***P<0.001$, *t* test on unpaired variables; ND: not determined.

FMLP-stimulated neutrophils was accompanied by a large and concentration-dependent increase of ATP secretion (Figure 5). No ATP release was detected 30 s after FMLP addition, then release reached a maximum at 1.5 min. The intensity of platelet activation (aggregation and ATP secretion) induced by neutrophils stimulated by 0.01 μM FMLP was comparable to that triggered by 0.1 nM Paf-acether (results not shown).

When mixtures of washed rabbit platelets and neutrophils were stimulated in the whole blood aggregometer, the addition of FMLP was followed by increased aggregation as measured by electrical impedance, with a time lag of approximatively 1 min and a plateau at 5 min (results not shown) and by a very intense decrease in platelet counts (Table 3). The increase of aggregation during platelet and neutrophil interaction was maximal with 0.01 μM FMLP, persisted at 1 nM and was absent at 0.1 nM (results not shown). The addition of FMLP to purified neutrophils examined with the whole blood aggregometer triggered a weak aggregation and a concentration-dependent decrease of neutrophil counts, which peaked at 0.01 μM (Table 3).

Release of enzymes

Platelets and neutrophils stimulated during 30 min at 37°C by 1 μM FMLP released respectively 2.2 ± 0.3% and 6.9 ± 1.9% of LDH, a figure comparable to basal release by unstimulated cells (4.4 ± 0.2%).

The release of lysozyme, a marker enzyme for neutrophil specific and azurophile granules, and of β-N-acetylglucosaminidase (NAGA) an enzyme contained in azurophile granules of neutrophils and in α granules of platelets was next studied. Lysozyme was not recovered from platelet supernatants or pellets, indicating the absence of neutrophil contamination. In contrast, when stimulated for 30 min with FMLP, neutrophils dose-dependently released lysozyme (36.6 ± 4.0 *vs.* 18.0 ± 4.9% of release for 0.1 μM FMLP, $n = 4-7$) and NAGA (29.0 ± 1.5 *vs.* 22.9 ± 2.8% of release for 0.1 μM FMLP, $n = 4-7$). These amounts were not significantly different from those detected when platelets were co-incubated with the neutrophils (34.2 ± 2.2 *vs.* 11.5 ± 1.3% of release of lysozyme and 23.9 ± 4.4 *vs.* 16.8 ± 1.9 of release of NAGA for 0.1 μM, $n = 4-7$).

Effect of potential inhibitors on platelet activation induced by FMLP-stimulated neutrophils

Inhibitors and antagonists were used to determine whether known mediators account for neutrophil-induced platelet activation. Aspirin and indomethacin, two cyclo-oxygenase inhibitors (both at 100 μM), BW 755C, a mixed cyclo-oxygenase-lipoxygenase

Table 4 Inhibition (%) of platelet-activation induced by neutrophils stimulated with 0.01 μM FMLP

Potential inhibitor	*μM*	*Pretreatment with neutrophils* Aggregation	*Pretreatment with neutrophils* Secretion	*Pretreatment with platelets* Aggregation	*Pretreatment with platelets* Secretion
Anti-cyclo-oxygenase					
Aspirin	100	30.0 ± 7.6	58.3 ± 8.7*	13.0 ± 6.6	38.3 ± 20.9
Indomethacin	100	34.0 ± 3.5*	48.0 ± 10.4	30.7 ± 8.5*	40.3 ± 5.2
Anti-lipoxygenase					
BW 755C	100	64.8 ± 9.2*	73.5 ± 13.5	44.0 ± 12.0	81.0 ± 7.6**
Paf-acether antagonist					
BN 52021	100	68.8 ± 7.9**	80.6 ± 5.5*	78.2 ± 7.2***	83.6 ± 3.2*
BN 52021	10	48.7 ± 15.8	61.3 ± 8.0	48.0 ± 13.0*	56.3 ± 10.2
WEB 2086	1	79.8 ± 9.6*	81.0 ± 6.1	75.0 ± 7.6**	80.3 ± 3.7
WEB 2086	0.01	15.3 ± 5.2	6.0 ± 3.5	17.8 ± 5.5	24.3 ± 6.3*

Washed rabbit platelets (0.2 ml; 10×10^8 per ml) or isolated neutrophils (0.2 ml; 10×10^6 per ml) were preincubated for 10 min at room temperature with aspirin and for 1 min at 37°C with indomethacin, BW 755C BN 52021 or WEB 2086. The other cell species (0.2 ml) was then added and the mixture was stimulated with 0.01 μM FMLP. Three minutes after adding FMLP, a 20 μl aliquot was collected for ATP determination (nmol ml^{-1}). Aggregation expressed as percentage of light transmission was measured 3 min after the addition of FMLP. The results are mean ± s.e.mean of 3–5 experiments. $^*P<0.01$; $^{**}P<0.05$; $^{***}P<0.001$, t test on unpaired variables.

inhibitor (100 μM) (Higgs *et al.*, 1979) and two Paf-acether antagonists, BN 52021 (100 and 10 μM) (Braquet *et al.*, 1985) and WEB 2086 (Casals-Stenzel *et al.*, 1986) (1 and 0.01 μM), were used. The inhibitors were incubated either with neutrophils, to inhibit the formation of mediator(s), or with platelets, to suppress the platelet effect of neutrophil-borne mediator(s). As shown in Table 4, pretreatment of neutrophils with aspirin and indomethacin reduced FMLP-induced platelet aggregation by about 30% and secretion by 50–60%, whereas BW 755C strongly decreased platelet activation. BN 52021 at 100 μM blocked platelet aggregation and secretion up to 70% and was less effective at 10 μM. At 0.01 μM, WEB 2086 almost suppressed platelet aggregation but had little effect on ATP secretion. In the same experimental conditions, 100 μM of BN 52021 and 1 μM of WEB 2086 totally inhibited platelet aggregation induced by 1 nM Paf-acether. When the inhibitors were incubated with platelets before the addition of FMLP-stimulated neutrophils, the percentages of inhibition observed were comparable or slightly reduced in comparison with those observed after preincubation with neutrophils (Table 4). Pretreatment of neutrophils for 10 min at 37°C with superoxide dismutase and catalase (20 μg ml^{-1} of each) had no effect on platelet aggregation or secretion.

Effect of a cell-free supernatant from FMLP-stimulated neutrophils

As shown in Figure 6, the supernatants from rabbit neutrophils stimulated for 3 min with 1 μM FMLP induced platelet aggregation and secretion only when BSA (0.25%, w/v) was present during neutrophil stimulation. Platelet activation induced by supernatants from FMLP-stimulated neutrophils was proportional to the number of activated neutrophils and was suppressed when platelets were preincubated with 100 μM BN 52021. These observations suggest the involvement of Paf-acether, since albumin has a stabilizing effect on Paf-acether activity (Benveniste *et al.*, 1972) and facilitates its release from activated neutrophils (Ludwig *et al.*, 1985) and its activity on platelets (Fouque, personal communication).

Formation of Paf-acether by FMLP-stimulated neutrophils

After stimulation by 1 and 0.01 μM FMLP in the presence of 0.25% BSA, neutrophils formed 1.0 and 1.1 nM of Paf-acether respectively of which only 50% were released to the extracellular medium (mean of 2 experiments in duplicate). In the absence of BSA, all Paf-acether remained associated to the neutrophils (0.45 and 0.58 nM Paf-acether formed with 1 and 0.01 μM FMLP respectively).

Morphology of platelets and neutrophils during interaction in the presence of FMLP

Control washed rabbit platelets were round and disk-shaped with few pseudopodia and showed the presence of dense bodies, α granules and the open canicular system (Figure 7 (1)). Washed rabbit platelets were unaffected by the 3 min incubation with

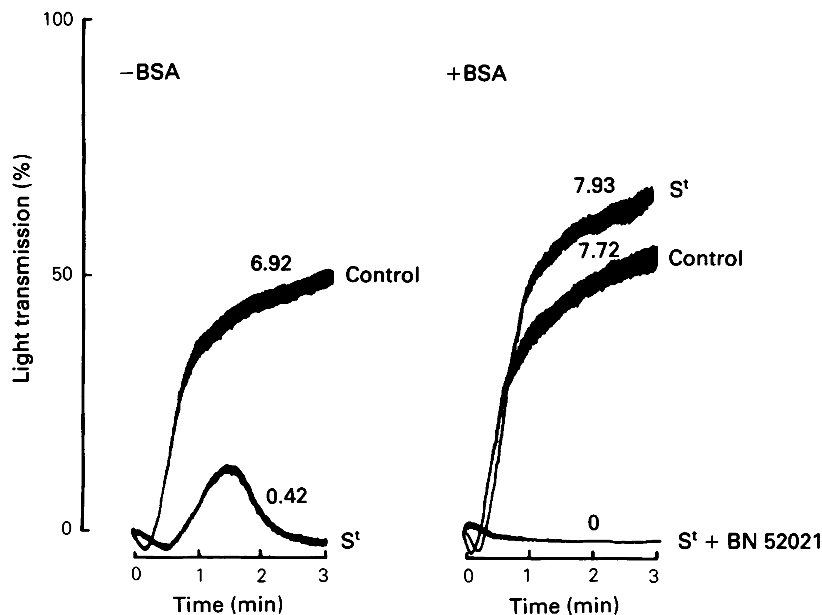

Figure 6 Activation of rabbit platelets by cell-free supernatants from neutrophils stimulated by FMLP in the presence or absence of bovine serum albumin (BSA). Rabbit neutrophils (1 ml; 10×10^6 per ml) were stimulated for 3 min with 1 μM FMLP in the presence or absence of 0.25% BSA. Aliquots (0.2 ml) were collected before (control) and 5 min after centrifugation (S^t). Platelets (0.2 ml, 10×10^8 per ml) were preincubated at 37°C either alone or in the presence of 100 μM BN 52021. Three min after the addition of the cell-free supernatant (0.2 ml), a 20 μl aliquot was collected for ATP determination. The numbers above the curves indicate ATP secretion (nM). The results are representative of 3 experiments.

FMLP (1 μM) as seen under electron microscopy (Figure 7 (2)). By contrast, FMLP induced aggregation of neutrophils (Figure 7 (4)). When washed rabbit platelets and purified neutrophils were co-incubated without FMLP, the two cell types were intact and not aggregated (Figure 7 (5)), but when 1 μM FMLP was added to the mixture of platelets and neutrophils, neutrophils were surrounded with aggregated and degranulated platelets (Figure 7 (6)).

Discussion

Different mechanisms of platelet-leukocyte interactions have been described. Thus, the products of the respiratory burst occurring during phagocytosis, i.e., superoxide anions and hydrogen peroxide, can modify platelet activity (Levine *et al.*, 1976; Clark & Klebanoff, 1980; Zoucas *et al.*, 1985). Villa *et al.* (1981) have shown that human blood leukocytes *in vitro* generate an inhibitor of platelet aggregation. Hällgren & Venge (1976) demonstrated that chymotrypsin-like cationic proteins of human granulocytes induce platelet aggregation and 5-hydroxytryptamine release. More recently, Chignard *et al.* (1986) have reported that a (chymotrypsin-like) protease, which they named neutrophilin, accounts for activation of human platelets by stimulated neutrophils. By contrast, elastase, the major neutral protease released by human polymorphonuclear leukocytes during coagulation or activation by soluble immune complex, by $C5_a$ or by endotoxin, inhibits thrombin-induced platelet activation (Brower *et al.*, 1985). Finally, Henson (1970) noted that the presence of neutrophils during the reaction of platelets with immune complexes or opsonized zymosan increased platelet secretion.

In this study, we have shown that the stimulation of neutrophils by FMLP induces platelet activation both in whole blood and isolated cells. Indeed, the addition of FMLP to rabbit blood was followed by increased electrical impedance, by a marked decrease in platelet counts and by a smaller decrease in leukocyte counts. When both cell types were isolated and co-incubated, FMLP triggered platelet aggregation and secretion and a decrease in cell counts, as well as aggregation of purified neutrophils accompanied by release of

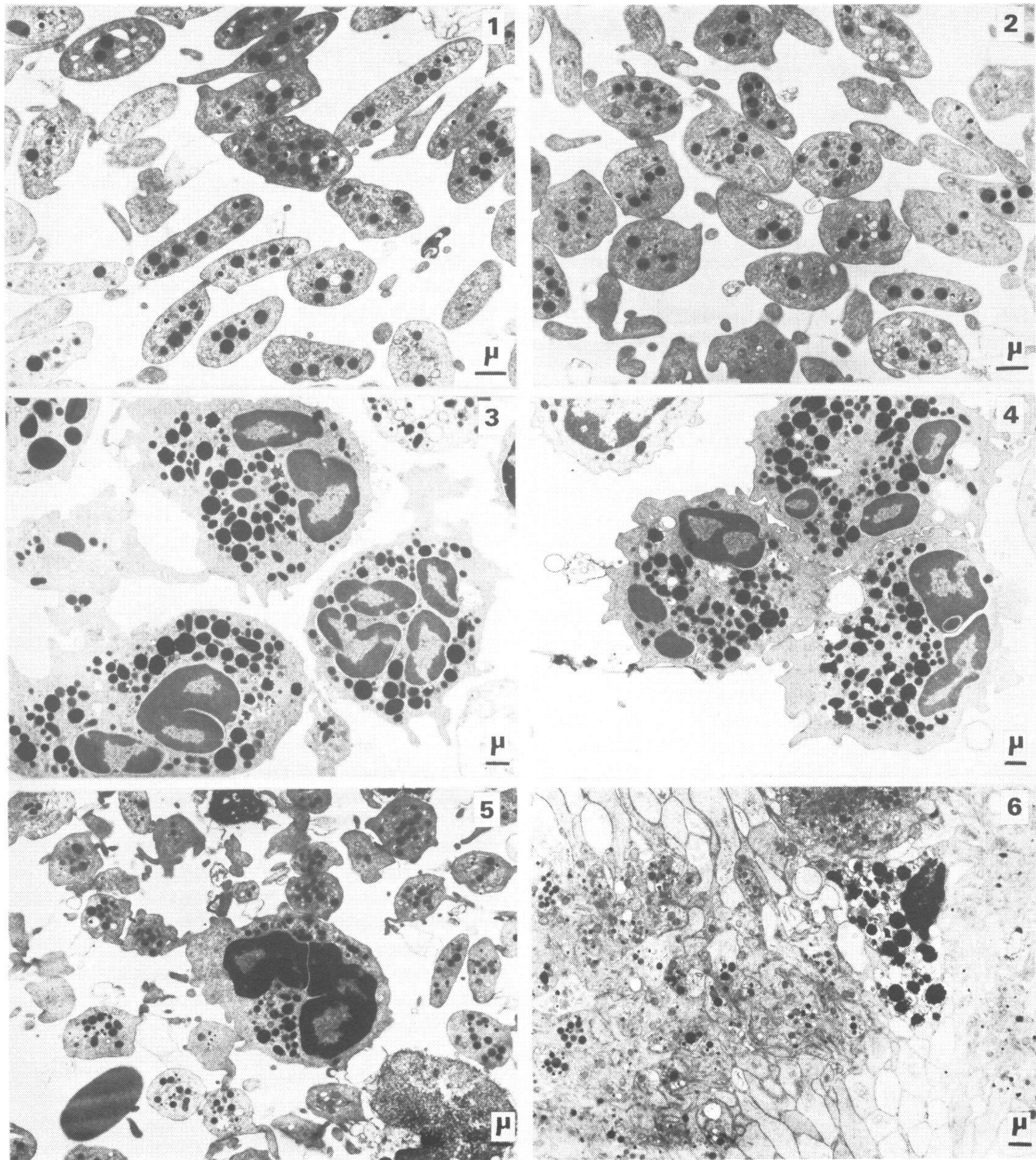

Figure 7 Transmission electron micrographs of washed rabbit platelets and purified neutrophils: (1) control washed rabbit platelets are round and disc-shaped with few pseudopodia and show the presence of dense bodies, alpha granules and the open canicular system (× 6000); (2) washed rabbit platelets are unaffected by incubation for 3 min with 1 μM FMLP (× 6000); (3) control purified neutrophils with nuclear lobes and azurophil and specific granules (× 4000); (4) neutrophils stimulated with 1 μM FMLP during 3 min are aggregated (× 4000); (5) washed rabbit platelets and purified neutrophils are co-incubated in the absence of FMLP (× 4000); (6) washed rabbit platelets and purified neutrophils are co-incubated and stimulated by 1 μM FMLP. The neutrophil is surrounded with aggregated and degranulated platelets (× 4000).

lysozyme. The active nature of the process was confirmed by the absence of platelet lysis as observed when lactic dehydrogenase release was evaluated. In contrast, FMLP lacked a direct platelet-stimulating effect.

The ultrastructural studies confirmed that both cells were intact before and after exposure, alone or associated, to FMLP, platelets being free and neutrophils forming aggregates, with no visible membrane fusion. These micrographs also confirmed that variations of impedance and of cell counts when mixed neutrophil-platelet suspensions were exposed to FMLP, do indeed reflect the formation of a neutrophil-platelet aggregate. In this aggregate, the platelets are markedly activated (degranulated), probably by Paf-acether formed by the neutrophils (see below), whereas the neutrophils are less affected.

Antagonism of the increased impedance in whole blood by the chemotactic peptide antagonist Boc-MLP (Spisani *et al.*, 1986) indicates that FMLP interacts with specific receptor sites on the neutrophil, with the formation of a platelet-activating substance. Failure of aspirin and indomethacin to inhibit FMLP-induced aggregation both in whole blood and in co-incubates of purified platelets and neutrophils ruled out a major role for cyclo-oxygenase-dependent metabolites of arachidonate, particularly of thromboxane A_2 released by stimulated neutrophils (Higgs *et al.*, 1976). By contrast, the lipoxygenase-dependent metabolites may be involved since Nafazatrom, a lipoxygenase inhibitor (Honn & Dunn, 1982), and BW 755C, a mixed anti-cyclo-oxygenase/anti-lipoxygenase agent (Higgs *et al.*, 1979) inhibited aggregation induced by FMLP in whole blood and in co-incubates of purified cells. This suggested that lipoxygenase-dependent arachidonic acid metabolites participate in platelet activation induced by neutrophils products after stimulation by FMLP.

Another candidate for mediating platelet-leukocyte interaction is Paf-acether, initially described in the context of immediate hypersensitivity (Benveniste *et al.*, 1972) and identified as 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine (Benveniste *et al.*, 1979; Demopoulos *et al.*, 1979). Paf-acether is formed by various cell types, including neutrophils (Lynch *et al.*, 1979; Jouvin-Marche *et al.*, 1984) and displays a large spectrum of biological activities; in particular, it is a potent platelet and neutrophil agonist (Benveniste & Vargaftig, 1983). Two of its antagonists, BN 52021 (Braquet *et al.*, 1985) and WEB 2086 (Casals-Stenzel *et al.*, 1986), suppressed altogether the increased electrical impedance in whole blood and activation of platelets by FMLP-stimulated neutrophils, pointing to the involvement of Paf-acether in platelet activation induced by FMLP in the presence of neutrophils.

Our preliminary attempts to demonstrate the release of soluble factor(s) from the FMLP-stimulated neutrophils in order to explain platelet activation were relatively unsuccessful, since the effects of supernatants from FMLP-stimulated neutrophils lose activity after 1 min, indicating their short lives. Nevertheless, neutrophil supernatants were stabilized and induced platelet activation when stimulation with FMLP was performed in the presence of albumin, in agreement with the fact that albumin stabilizes Paf-acether (Benveniste *et al.*, 1972). In this respect, it has been shown that human platelets require the presence of albumin to support Paf-acether-induced aggregation, which furthermore is reduced when albumin is replaced by gelatin (Fouque *et al.*, unpublished results). When stimulated by FMLP in the presence of albumin, neutrophils formed approximately 1 nM Paf-acether, half of it being released to the extracellular medium. In contrast, in the absence of albumin, all Paf-acether formed remained cell-associated. Thus a Paf-acceptor molecule such as albumin is required to facilitate the release of Paf-acether from stimulated cells and indeed, Ludwig *et al.* (1985) reported that increased albumin concentrations promote increased Paf-acether synthesis and release by FMLP-stimulated human neutrophils in the presence of cytochalasin B. In our experiments, similar results with rabbit neutrophils were obtained in the absence of cytochalasin B and rabbit neutrophils stimulated by opsonized zymosan formed 90, 1360 and 15300 ng ml^{-1} Paf-acether in the presence of 0, 2.5 and 50 mg ml^{-1} rabbit albumin, respectively (Coëffier *et al.*, unpublished results). Thus, the majority of newly synthesized Paf-acether appears to be retained within the cell and is not released (Lynch & Henson, 1986). In the presence of albumin, the Paf-acether formed by FMLP-stimulated rabbit neutrophils was also more easily released and the possibility that it could activate surrounding platelets was thus increased. Furthermore, albumin protected Paf-acether against degradation, since we noted that the activation of platelets disappeared with time after incubation of supernatant at 37°C alone or with neutrophils. In similar conditions, it has also been shown that addition of platelets to neutrophils causes an increased synthesis of Paf-acether, neutrophils acetylating the excess of deacetylated Paf-acether released by human platelets (Benveniste & Coëffier, 1984; Coëffier *et al.*, 1984).

Our results suggest that both lipoxygenase-dependent arachidonic acid metabolites and Paf-acether are involved in activation of platelets during platelet-leukocyte interaction after stimulation by FMLP. These data are in agreement with the results of Henson (1981) and of Oda *et al.* (1986). That this newly described mechanism for platelet-neutrophil interaction may be important under *in vivo* conditions, is highlighted by the results in whole blood, since, contrary to the stimulation with human neutrophils,

no cytochalasin B was required to allow mixed aggregate formation.

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