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Direct evidence for the existence of a neutrophil-derived platelet activator (neutrophilin)

(cell–cell interactions/calcium mobilization/platelet activation/*N*-formyl-Met-Leu-Phe/serine protease)

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ABSTRACT **Human neutrophils and platelets were loaded with the intracellular calcium indicator fura-2. The chemotactic peptide *N*-formyl-Met-Leu-Phe (fMet-Leu-Phe) induced a rapid elevation of cytosolic free calcium in cytochalasin B-treated neutrophils but failed to increase the cytosolic calcium in platelets. On the other hand, when unloaded neutrophils were incubated together with autologous fura-2-loaded platelets, fMet-Leu-Phe stimulated a 6-fold increase in platelet cytosolic calcium subsequent to a brief lag. Parallel experiments demonstrated that the addition of fMet-Leu-Phe to neutrophil/platelet incubates also elicited platelet aggregation and serotonin release. Platelet activation showed a positive correlation with the concentration of fMet-Leu-Phe added to the mixed cell population. Cell-free supernatants prepared from fMet-Leu-Phe-stimulated neutrophils were capable of inducing platelet calcium mobilization, aggregation, and secretion. The amount of platelet-activating material present in the supernatant was proportional to the number of activated neutrophils. Preincubation of platelets with BN 52021, acetylsalicylic acid, SQ-29,548, or hirudin did not modify the aggregation response induced by the supernatant collected from fMet-Leu-Phe-activated neutrophils, suggesting that the material was not 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (paf-acether), arachidonic acid, thromboxane A2, or thrombin. Pretreatment of the neutrophil supernatant with an ADP (creatine phosphate/creatine phosphokinase) or a superoxide/peroxide (superoxide dismutase/catalase) scavenging system also had no effect on aggregation or secretion, indicating that these substances did not participate in platelet activation. The biological activity present in the neutrophil supernatant was destroyed by heat and inactivated by treatment with phenylmethylsulfonyl fluoride, indicating that it is a protein and most probably an enzyme with serine protease activity. These data provide the direct observation of secondary signal transmission to platelets following primary activation of neutrophils. We propose the name neutrophilin for the neutrophil-derived mediator.**

Platelets and neutrophils are known to participate in different inflammatory disease processes. For example, it is now generally agreed that the mechanism of pathogenesis of the adult respiratory distress syndrome involves activation of leukocytes and platelets (1). Using an *in vivo* experimental model of acute lung injury, it has been shown that neutrophil or platelet depletion resulted in a marked reduction of the extent of the injury (2). Furthermore, intradermal injection of an inflammatory stimulus such as *N*-formyl-Met-Leu-Phe (fMet-Leu-Phe) triggered leukocyte and platelet accumulation at the inflammatory site. Interestingly, neutrophil depletion prevented platelet deposition in response to the inflammatory stimulus, suggesting a central role for neutrophils (3). It was speculated that paf-acether might provide a possible link between platelets and neutrophils (3) and this assumption has been reinforced by the recent observation that neutrophils and platelets cooperate *in vitro* in paf-acether formation (4). It is well known that these two cell types also cooperate in the metabolism of arachidonic acid, another important mediator of inflammation (5–7). However, these different *in vitro* studies have not shown the release of a factor, assumed to be paf-acether, by activated neutrophils that could initiate platelet activation. The current investigation was undertaken to determine if neutrophils do release a factor(s) capable of activating platelets and, if so, to determine the nature of the substance responsible for that activation. The data presented here demonstrate that fMet-Leu-Phe stimulation of neutrophils releases a factor or factors that is fully capable of rapidly inducing platelet calcium mobilization, secretion, and aggregation (platelet activation). This factor is neither paf-acether nor a metabolite of arachidonic acid but is an unidentified protein. This phenomenon could have important implications in the onset and propagation of the inflammatory process *in vivo* in which the participation of neutrophils and platelets has been demonstrated but for which the mediator and mechanism involved have not yet been defined.

MATERIALS AND METHODS

Preparation of Platelets. Human blood was collected from healthy volunteers who had not taken aspirin during the previous 10 days and was treated with acid/citrate/dextrose as anticoagulant (8). Platelet-rich plasma (PRP) was prepared by centrifugation at 180 × *g* for 20 min. PRP was concentrated at 500 × *g* for 20 min and the resulting platelet pellet was resuspended in 0.5 vol of the platelet-poor plasma. Fura-2 acetoxymethyl ester dissolved in dimethyl sulfoxide was incubated with the concentrated PRP at a final concentration of 3 μM at 37°C for 30 min. When loading was complete, the platelets were collected by centrifugation at 500 × *g* for 20 min and gently resuspended in Tyrode's buffer supplemented with 10 mM glucose (pH 7.4). The platelet concentration was adjusted to 4×10^8 cells per ml and kept at ambient temperature. For serotonin release studies, PRP was incubated at 37°C for 30 min with 1 μM [^{14}C]serotonin (55 mCi/mmol; 1 Ci = 37 GBq) and then centrifuged and processed as described except that 2 μM imipramine was added to the final cell suspension.

Preparation of Neutrophils. Human blood was collected using acid/citrate/dextrose as anticoagulant (8). Two volumes of whole blood were mixed with 1 vol of 3% (wt/vol)

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Abbreviations: fMet-Leu-Phe, *N*-formyl-Met-Leu-Phe; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; paf-acether, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine or PAF.

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dextran in saline and erythrocytes were allowed to sediment for 30 min at room temperature. The upper leukocyte-enriched plasma was collected and gently layered over Histopaque 1077 and the gradient was centrifuged at 350 × *g* for 45 min. The cell pellet was resuspended in an erythrocyte lysis buffer composed of 155 mM NH_4Cl, 2.96 mM $KHCO_3$, and 3.72 mM EDTA. The tube was gently inverted and after 5 min the suspension was centrifuged at 350 × *g* for 10 min and the cell pellet was washed in excess Hanks' balanced salt solution lacking magnesium and calcium. Cell number and purity were determined following dilution in Turk's solution and viability was assessed by trypan blue dye exclusion. Unless otherwise stated, the cells were resuspended at a concentration of 10^7 per ml and the cell preparation was maintained at ambient temperature in the presence of 1 mM $MgCl_2$. Cells prepared in this manner routinely contained 98 ± 1% neutrophils and were 97 ± 2% viable. In some experiments purified neutrophils were incubated for 30 min at 37°C with 1.5 μM fura-2 acetoxymethyl ester in the presence of 1 mM $MgCl_2$ and 1 mM $CaCl_2$ prior to the final centrifugation.

Fluorescence Measurements and Estimation of Cytosolic Calcium. Aliquots (1.25 ml) of fura-2-loaded platelets and unloaded neutrophils were preincubated together for 5 min in the presence of 1.3 mM $CaCl_2$ and 5 μg of cytochalasin B per ml. In some experiments fura-2-loaded neutrophils or fura-2-loaded platelets were preincubated with 1.25 ml of buffer. Fluorescence measurements were conducted in a Perkin-Elmer model LS-5 fluorometer using a quartz cuvette thermostatically controlled at 37°C and continuously stirred. Fura-2 fluorescence signals were obtained using excitation wavelengths of 340 nm and 380 nm (5 nm slit width) and an emission wavelength of 510 nm (10 nm slit width). Dual wavelength recordings of the 340 nm/380 nm excitation ratio were generated by driving the single monochrometer back and forth between 340 nm and 380 nm. Cytosolic free calcium concentrations were calculated from the 340 nm/380 nm ratios according to Grynkiewicz *et al.* using a K_d of 224 nM (9).

Measurement of Platelet Aggregation and Secretion. [^{14}C]Serotonin-loaded platelets (250 μl) were incubated with Tyrode's buffer containing glucose (250 μl) at 37°C while stirring for 5 min in the presence of 1.3 mM $CaCl_2$ and 160 μg of fibrinogen per ml. Aliquots, as indicated, of fMet-Leu-Phe-activated neutrophil supernatant were added to radiolabeled platelets and aggregation was monitored by determining changes in light transmission using a Payton aggregometer. Three minutes after addition of the supernatant, the entire sample was added to 125 μl of a stopping solution composed of 33% formaldehyde, 77 mM EDTA, and 155 mM NaCl (1:9:8 volumes, respectively). Following a 2-min centrifugation at 13,000 × *g* in a microcentrifuge, 400 μl of the supernatant was added to 10 ml of scintillation fluid (Liquiscint, National Diagnostics, Somerville, NJ). In each set of experiments the total serotonin content of platelets was measured by adding 500 μl of the platelet suspension to the stopping solution, of which 400 μl was transferred directly to the counting solution. Serotonin release was expressed as percent of the total serotonin content (assumed to be 100%) and aggregation was expressed as percent of the maximal light transmission. In some experiments, 250 μl of platelets (4 × 10^8 per ml) was mixed with 250 μl of neutrophils (10^7 per ml) in the presence of 5 μg of cytochalasin B per ml and 1.3 mM $CaCl_2$ while stirring at 37°C. Five minutes later the incubate was challenged with fMet-Leu-Phe for aggregation and serotonin release measurements.

Lactate Dehydrogenase Assay. Platelet suspensions were incubated as described for aggregation and secretion. Three minutes after the addition of neutrophil supernatant the cells were centrifuged at 13,000 × *g* for 2 min. The extracellular medium was retained and assayed within 3 hr for the presence of lactate dehydrogenase. Lactate dehydrogenase was measured at 37°C in the presence of 120 mM phosphate buffer (pH 7.3), 0.18 mM NADH, and 0.91 mM pyruvate. The decrease in absorbance at 340 nm was recorded against a blank composed of phosphate buffer. Lactate dehydrogenase release was expressed as percent of the total lactate dehydrogenase present in supernatants from platelets lysed with 0.2% (vol/vol) Triton X-100 followed by a single cycle of freeze thawing.

Reagents. Cytochalasin B, fMet-Leu-Phe, phenylmethylsulfonyl fluoride ($PhMeSO_2F$), fibrinogen, epinephrine, acetylsalicylic acid, hirudin, Histopaque 1077, catalase, superoxide dismutase, creatine phosphate, and creatine phosphokinase were obtained from Sigma. Fura-2 acetoxymethyl ester was purchased from Molecular Probes (Eugene, OR) and paf-acether was from Avanti Polar Lipids. [^{14}C]Serotonin was obtained from Amersham and ZK36374 was from Berlex (a subsidiary of Schering). BN 52021 was the generous gift of P. Braquet (IHB-IPSEN, France), and SQ-29,548 was kindly provided by D. Harris (Squibb).

RESULTS

Neutrophils loaded with fura-2 and stimulated by the chemotactic peptide fMet-Leu-Phe in the presence of cytochalasin B and exogenous calcium rapidly underwent an elevation in cytosolic free calcium. At 250 nM fMet-Leu-Phe, the cytosolic calcium concentration rose from a resting level of 52 nM ± 7 nM to a peak value of 355 nM ± 52 nM (mean ± SD; n = 4). The addition of the same concentration of fMet-Leu-Phe to fura-2-loaded platelets did not increase the cytosolic calcium concentration, confirming that the chemotactic peptide is unable to stimulate platelets. The subsequent addition of paf-acether did, however, induce an increase in cytosolic calcium, verifying that the platelets are responsive to stimulatory agonists (Fig. 1). When a mixture of fura-2-loaded platelets and unloaded autologous neutrophils was stimulated with 250 nM fMet-Leu-Phe, the platelet cytosolic calcium rose after a lag of ≈10 sec from a basal level of 73 nM ± 11 nM to a peak calcium of 441 nM ± 78 nM within 60 sec (mean ± SD; n = 11). Fig. 1 shows a representative tracing of calcium mobilization observed in a mixture of platelets and

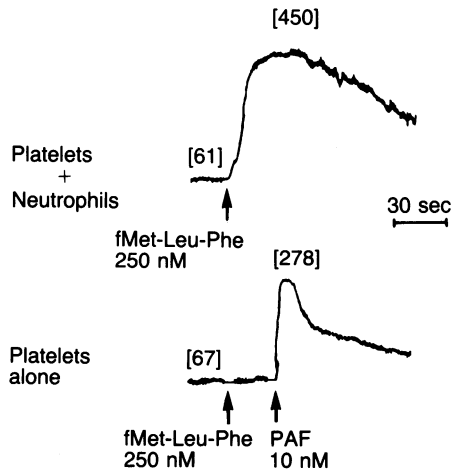

FIG. 1. Calcium mobilization in platelets induced by addition of a neutrophil-specific agonist, fMet-Leu-Phe. Cell suspensions were preincubated for 5 min at 37°C in the presence of 1.3 mM $CaCl_2$ and 5 μg of cytochalasin B per ml prior to addition of fMet-Leu-Phe or paf-acether (PAF). Numbers in brackets indicate the resting and peak cytosolic free calcium concentrations expressed in nM and calculated from the 340 nm/380 nm fura-2 ratios. Shown are the 340 nm fura-2 fluorescence signals.

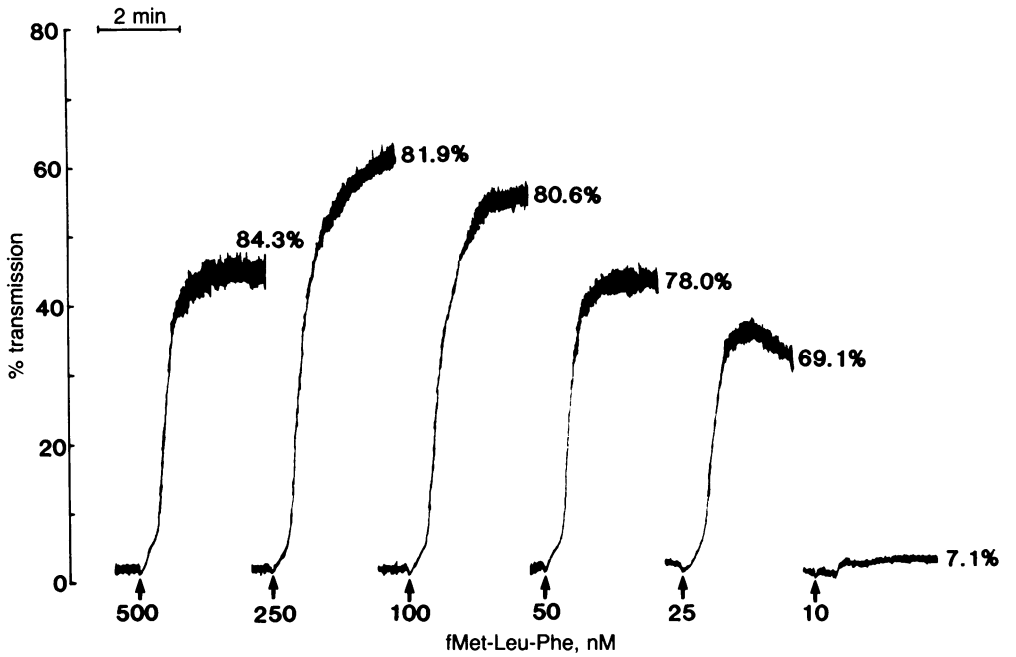

FIG. 2. Platelet aggregation and serotonin release induced by addition of fMet-Leu-Phe to an incubate of platelets and neutrophils. Cell suspensions were preincubated for 5 min at 37°C in the presence of 1.3 mM $CaCl_2$, 5 μg of cytochalasin B per ml, and 160 μg of fibrinogen per ml prior to addition of the designated concentrations of fMet-Leu-Phe. Aggregation was monitored continuously, whereas the percent [^{14}C]serotonin secretion indicated at the end of each trace refers to the extent of release after 3 min.

neutrophils. The amount of calcium mobilized by platelets increased linearly as the concentration of fMet-Leu-Phe was varied between 5 and 250 nM. Parallel mixed cell population experiments demonstrated that 250 nM fMet-Leu-Phe also induced platelet aggregation and serotonin release. The extent of platelet secretion and aggregation was dependent on the fMet-Leu-Phe concentration with which the neutrophils were challenged (Fig. 2).

To ascertain whether a soluble neutrophil factor (or factors) was responsible for platelet activation, a cell-free supernatant was prepared 3 min after fMet-Leu-Phe stimulation of neutrophils. As seen in Fig. 3, increasing volumes of supernatant induced progressively greater serotonin release and aggregation responses. Similar to many other platelet agonists, platelet aggregation by supernatant was dependent on the presence of fibrinogen and extracellular calcium (5

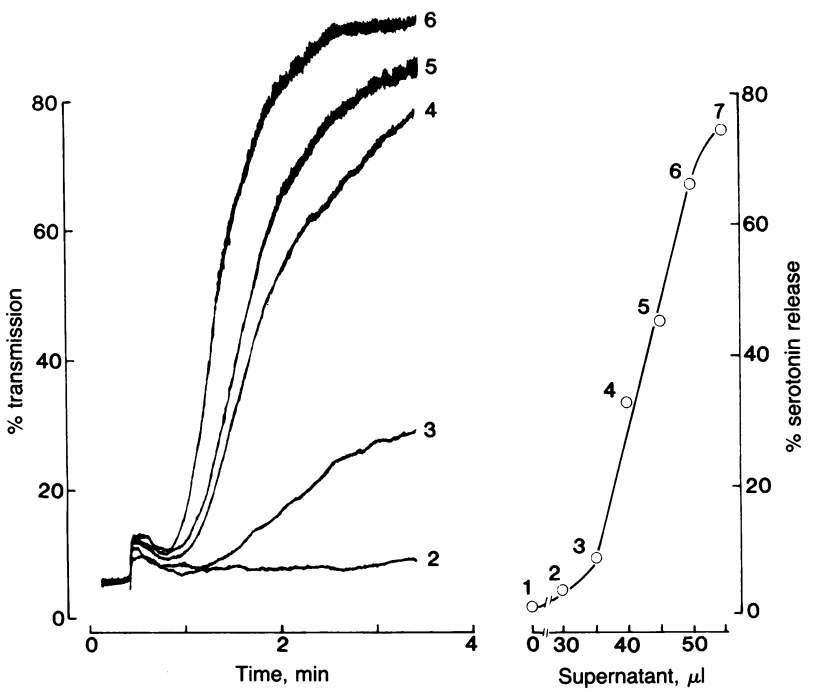

FIG. 3. Correlation between platelet aggregation and secretion and the volume of supernatant added from fMet-Leu-Phe-stimulated neutrophils. A cell-free supernatant was prepared 3 min after the addition of 500 nM fMet-Leu-Phe to a suspension of 3×10^7 neutrophils per ml. Platelets were preincubated for 2 min at 37°C with 1.3 mM calcium and 160 μg of fibrinogen per ml prior to addition of the designated volumes of supernatant. (*Right*) Percent [^{14}C]serotonin release 3 min after addition of the indicated volumes of supernatant. (*Left*) Platelet aggregation obtained with the corresponding volumes of supernatant.

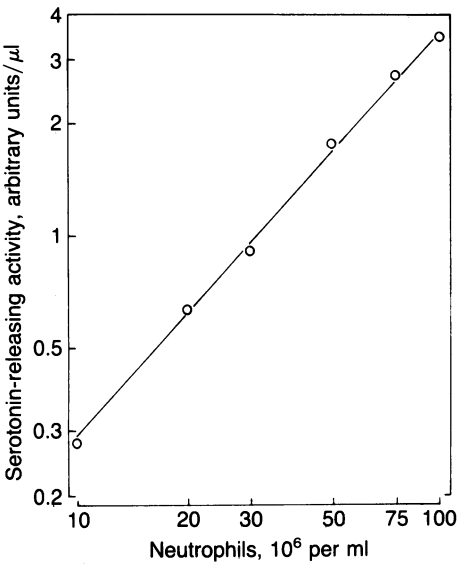

FIG. 4. Relationship between the number of fMet-Leu-Phe-activated neutrophils and the amount of serotonin-releasing activity present in the supernatant. Cell-free supernatants were prepared 3 min following addition of 1 μM fMet-Leu-Phe to suspensions of neutrophils. The volume of each supernatant capable of releasing 50% of the total serotonin content of platelets was determined and assumed to represent 50 arbitrary units. From this value the corresponding arbitrary units/μl were calculated and plotted as a function of each cell concentration.

mM EGTA totally suppressed platelet responses). Furthermore, platelet activation was totally suppressed by the prostacyclin analogue ZK36374 (5 nM) and was enhanced by epinephrine (data not shown). Less than 1% of the total platelet lactate dehydrogenase was recovered in the extracellular medium 3 min after addition of neutrophil supernatant at a volume five times higher than a threshold volume capable of inducing a full aggregation and secretion. These observations provided conclusive proof that the neutrophil mediator was not mediating cytolysis.

Additional experiments showed that the neutrophil-derived platelet activator had achieved a maximal concentration in the supernatant of activated neutrophils within 1–3 min of fMet-Leu-Phe challenge and the level thereafter remained at a plateau for up to 10 min subsequent to stimulation. As seen in Fig. 4, the amount of biologically active material released from neutrophils and present in the supernatant was proportional to the number of cells activated by fMet-Leu-Phe.

The nature of the platelet activating material was tested by using different specific inhibitors. The inhibitors were tested for their effectiveness in blocking platelet activation by measuring aggregation and secretion induced by the specific agonist against which they were directed before being used at the required maximal inhibitory concentration against aliquots of the fMet-Leu-Phe-activated neutrophil supernatant. Thus, 100 μM BN 52021, a paf-acether antagonist (10), 100 μM acetylsalicylic acid, a platelet cyclooxygenase inhibitor (11), 50 μM SQ-29,548, a thromboxane A_2 antagonist (12), and 1 unit of hirudin per ml, a thrombin antagonist (13), were used. As shown in Table 1, platelet aggregation induced by the neutrophil-released substance was not affected by any of the inhibitors tested. Serotonin release, however, was partially inhibited by acetylsalicylic acid and SQ-29,548. Pretreatment of the neutrophil supernatant for 10 min with creatine phosphate/creatine phosphokinase or with superoxide dismutase/catalase had no effect on platelet aggregation or secretion. By contrast, incubation of the supernatant with $PhMeSO_2F$ for various periods of time at different concentrations led to complete suppression of the platelet aggregating and releasing activities (Fig. 5). The platelet-activating activity was totally destroyed by a 5-min incubation at 90°C.

DISCUSSION

Intravenous injection of fMet-Leu-Phe has been shown to induce thrombocytopenia as well as neutropenia in rabbits (3). Intrabronchial injection of C3a, another neutrophil-specific agonist, resulted in the formation of leukocyte and platelet aggregates in the pulmonary vessels and in the heart of guinea pigs (14). In both of these cases, thrombocytopenia was postulated to be caused by a secondary mediator released by neutrophils. The data presented here have clearly established that primary stimulation of neutrophils by fMet-Leu-Phe results in the release of a secondary signal that is transmitted to and is fully capable of activating platelets, data that could explain the *in vivo* observations previously mentioned. Upon stimulation by fMet-Leu-Phe, cytochalasin B-treated neutrophils released to the medium a soluble material that induced calcium mobilization, serotonin release, and aggregation of platelets. Although it is known that activated human neutrophils release different substances (15), only a few are known to affect platelets—i.e., paf-acether (16), thromboxane A_2, and arachidonic acid (17) and superoxide ions and peroxide (18, 19). Through the use of specific inhibitors, we have provided evidence that none of these substances was responsible for the observed platelet activation induced by the supernatant from fMet-Leu-Phe-

Table 1. Effect of different antagonists on platelet aggregation and secretion induced by the supernatant from fMet-Leu-Phe-stimulated neutrophils

Agent or treatment	% inhibition	
	Aggregation	Secretion
BN 52021 (100 μM)*	2.3 ± 2.7	2.5 ± 2.3
Acetylsalicylic acid (100 μM)*	0.6 ± 1.4	33.1 ± 13.2†
SQ-29,548 (50 μM)*	2.3 ± 1.6	34.1 ± 7.0†
Hirudin (1 unit/ml)*	3.8 ± 6.5	2.5 ± 2.3
Creatine phosphate (20 mM) + creatine phosphokinase (20 units/ml)‡	5.8 ± 7.8	2.9 ± 4.2
Superoxide dismutase (20 μg/ml) + catalase (20 μg/ml)‡	6.7 ± 1.7	1.7 ± 2.4

The percent inhibition represents the mean ± SD of three to five determinations. For each set of experiments, a volume of fMet-Leu-Phe-stimulated neutrophil supernatant was chosen that induced a subthreshold aggregation.

*Platelets were preincubated for 5 min at 37°C in the presence of 1.3 mM calcium, 160 μg of fibrinogen per ml, and each agent at the designated concentration prior to the addition of a cell-free supernatant prepared from neutrophils activated with fMet-Leu-Phe.

†Inhibition was significantly different from the control sample ($P < 0.05$; Student's t test).

‡Aliquots of fMet-Leu-Phe-stimulated neutrophil supernatant were preincubated for 10 min at 37°C with the scavenging system prior to addition to platelets preincubated with 1.3 mM calcium and 160 μg of fibrinogen per ml.

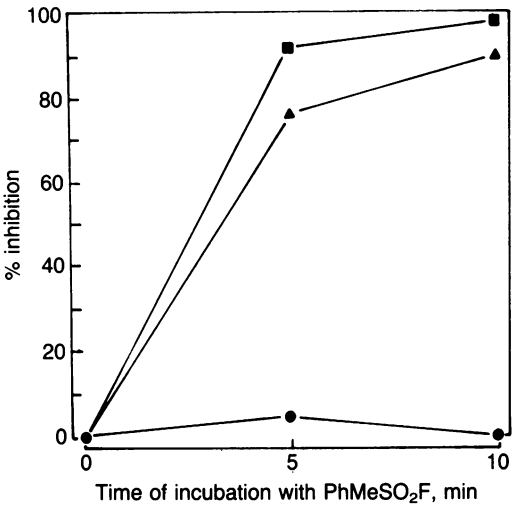

FIG. 5. Inhibition by $PhMeSO_2F$ of the platelet-releasing activity of fMet-Leu-Phe-activated neutrophil supernatant. Aliquots of supernatant were incubated at 37°C for various time intervals with three different concentrations of $PhMeSO_2F$ dissolved in dimethyl sulfoxide. The supernatant was subsequently added to platelet suspensions and the extent of serotonin release was measured after 3 min. The percent inhibition was determined by comparing secretion induced by the supernatant incubated in the presence of $PhMeSO_2F$ with a corresponding control sample incubated under similar conditions with dimethyl sulfoxide. ●, 40 μM; ▲, 50 μM; ■, 60 μM.

stimulated neutrophils. We have also shown that platelet activation could not be due to thrombin formation or to the presence of ADP. The lack of an effect of acetylsalicylic acid or SQ-29,548 on aggregation also eliminated a possible participation of collagen.

These data lead us to postulate that we are dealing with a different type of platelet activator. Nonetheless, platelet activation by the neutrophil factor conforms to a pattern seen with other classical platelet agonists—i.e., it is dependent on external calcium, requires fibrinogen, is inhibited by a prostacyclin analog, and is enhanced by threshold concentrations of epinephrine. Although aggregation was not modified by cyclooxygenase inhibitors, acetylsalicylic acid, or the thromboxane A_2 antagonist SQ-29,548, serotonin release was suppressed by ≈30%. These latter observations probably indicate that the endogenous arachidonate pathway is in part involved in the platelet activation process.

That the substance described was susceptible to $PhMeSO_2F$, a serine protease inhibitor, as well as to heat allows us to postulate that the factor responsible for platelet activation is an enzyme. Among the many proteins known to be released from stimulated neutrophils (20), only cathepsin G has been reported to possess platelet-activating properties (21). This chymotrypsin-like protease has been shown to induce platelet aggregation and secretion. It is thus possible that this enzyme is responsible for the activation of platelets we have observed. However, Bykowska and coworkers (21) observed that, at all concentrations used, a purified leukocyte cathepsin G induced aggregation with a lag phase longer than that observed when thrombin was used as the agonist. In our hands no difference between the onset of aggregation induced by supernatant or thrombin was noted. Regardless of its identity, this factor could explain different pathological events in which neutrophils and platelets are known to be involved, including lung (22) and arterial endothelial injury (23).

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