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HUMAN NEUTROPHIL-DERIVED PLATELET ACTIVATING FACTOR[1]

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Platelet-activating factor (PAF) is released from rabbit basophils by IgE-associated mechanisms and is a major mediator of acute allergic reactions in rabbits. Its structure has recently been suggested to be 1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine. Experiments were performed to determine whether liberation of a similar material could be demonstrated from human leukocytes. This study describes the release of a material from human neutrophils with properties very similar to rabbit basophil-derived PAF.

Human neutrophils from nonatopic subjects were purified and incubated with opsonized zymosan. The resultant supernatant reacted with washed rabbit platelets to induce aggregation and release of previously incorporated tritiated serotonin, and with human platelets to induce aggregation. With the use of appropriate inhibitors this activity was demonstrated to be unrelated to thrombin, ADP, or arachidonic acid. The supernatant was shown to be similar to rabbit PAF by several criteria: 1) the active factor bound to albumin; 2) the kinetics of rabbit platelet aggregation and serotonin release elicited by the supernatant were identical to that induced by PAF; 3) the activity was extractable into the chloroform phase of a chloroform:methanol:water system, indicating a lipid nature; 4) both materials demonstrated identical mobility patterns on thin-layer chromatography. The derivation of this activity from neutrophils may indicate a role for PAF as a mediator of nonallergic inflammatory reactions.

Platelet-activating factor (PAF)[2] is a soluble factor derived from rabbit leukocytes that has been shown to be a potent inducer of rabbit platelet aggregation (1–4) and of the release of the substances serotonin, histamine, ADP, and prostaglandins (1, 3, 5, 6). PAF has a low m.w. (7, 8), binds to albumin (7), and is thought to be phospholipid in nature (9). It has been demonstrated in the rabbit to be a mediator of experimentally induced anaphylaxis (10, 11), and most systems for effecting PAF release have used an IgE-related mechanism (antigen or anti-IgE) (3, 12). Abundant but circumstantial evidence has implicated the basophil as the cell of origin (13, 14). However, sensitized lung tissue also releases a platelet-activating factor (PAF_L), presumably derived from mast cells (15), and a recent report has suggested that a PAF-like material can be released from peritoneal macrophages (16). In recent studies (17), our collaborators in San Antonio have successfully synthesized a glycerol ether (1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorycholine) with identical physicochemical and functional properties to rabbit PAF derived from a) antigen stimulated basophils, b) rabbit neutrophils, and c) plasma from rabbits undergoing anaphylaxis. Since Clark *et al.* (18) have shown our human neutrophil-derived PAF to have identical physicochemical properties to the rabbit materials, this may indicate, in addition, the nature of the human material.

In contrast to the situation in the rabbit, attempts to demonstrate the existence of PAF in man have met with more variable success. Several reports have indicated the existence of human PAF, again presumably basophil-derived (7, 16, 19–21). Lewis *et al.* (20) showed that the leukocytes of a patient with basophilic leukemia, at least 75% of which were basophils, could generate PAF in the presence of the calcium ionophore A23187. Later Camussi *et al.* (21) reported that immune complexes, antigen, anaphylatoxins, and neutrophil-derived cationic proteins could also stimulate PAF release from normal human leukocytes. Although basophil degranulation was demonstrable coincident with PAF presence, the cell fractions used in these experiments were relatively impure.

More recently, we have been able to demonstrate the derivation of a material similar if not identical to PAF from rabbit polymorphonuclear neutrophils (22). This activity was generated when rabbit neutrophils were incubated with opsonized zymosan (ZC). In the current report, we have extended our studies to human leukocytes. Human polymorphonuclear neutrophils incubated with ZC were shown to produce a factor with potent effects on rabbit and human platelets. This substance was soluble in chloroform, probably lipid, and at present is simply designated as human neutrophil supernatant (H-N-SN) until its character can be further defined. Many of the properties of H-N-SN appear identical to those associated with antigen-derived PAF from rabbits (18).

MATERIALS AND METHODS

Animals. New Zealand White rabbits of both sexes weighing 2.5 to 4 kg were employed as the source of rabbit platelets. Blood for platelets was not removed at intervals of less than 1 month.

Human subjects. Healthy adult volunteers, taking no medications for at least 1 week, were used as a source of neutrophils and platelets.

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[1] This work was supported by United States Public Health Service, National Institutes of Health Grant HL-22555.
[2] Abbreviations used in this paper: PAF, platelet-activating factor; PAF_L, lung platelet-activating factor; ZC, opsonized zymosan; H-N-SN, human neutrophil supernatant; R-N-SN, rabbit neutrophil supernatant; EGTA, ethylene glycol tetraacetate; CP, creatine phosphate (phosphocreatine); CPK, creatine phosphokinase; LDH, lactic dehydrogenase; β-gluc, β-glucuronidase; MPO, myeloperoxidase; ^{3}H-5HT, tritiated serotonin; ETYA, eicosatetraenoic acid.

Preparation of human neutrophils. Human neutrophils were prepared with minor modifications of a technique previously described (23). Briefly, blood was drawn into 1/10 volume 3.8% citrate and centrifuged at 300 × G for 20 min at room temperature. Platelet-rich plasma was aspirated, and platelets were removed by centrifugation at 2500 × G for 15 min to provide platelet-poor plasma. Remaining blood was sedimented in Dextran T-500 (Pharmacia Fine Chemicals, Piscataway, N. J.), final concentration of 0.6%, at room temperature for 30 min. Supernatant cells were pelleted at 275 × G for 10 min and resuspended in platelet-poor plasma diluted 1:4 in saline. The cell suspension (8 ml) was layered over 3 ml Ficoll-Hypaque (Pharmacia, 1.077 g/ml) and centrifuged at 750 × G for 25 min at 18°C. Pelleted cells were resuspended in erythrocyte-lysing solution (23), centrifuged at 275 × G for 10 min, and washed twice in Tyrode's-BSA (see "Buffers"). Cells were resuspended to 1×10^7/ml in Tyrode's-BSA, stained in human basophil-eosinophil stain (24), and represented 92 to 98% neutrophils. Basophils were always less than 2/1000 cells, and often none were seen. Eosinophils represented 1.0 to 4.2% of cells present.

Buffers. a) Tyrode's solution: NaCl, 8 g/liter; KCl 0.195 g/liter; $NaHCO_3$, 1.02 g/liter; $MgCl_26H_2O$, 0.213 g/liter; $CaCl_2 \cdot 2H_2O$, 0.145 g/liter; glucose 1 g/liter, pH 7.3; b) Tyrode's-gelatin: same as "a" with 0.25% gelatin; c) Tyrode's-BSA: Same as "a" with 0.25% BSA (Fraction V, Sigma Chemical Co., St. Louis, Mo.); d) Tyrode's-gelatin-EGTA: Tyrode's-gelatin without Ca^{++} and with 0.1 mM ethylene glycol tetraacetate (EGTA), pH 6.5; e) Tyrode's-gelatin (or BSA) without Ca^{++}: same as "c" or "b" without Ca^{++}, pH 6.5. f) Tyrode's-gelatin with citrate: same as "b" with 0.38% trisodium citrate.

Preparation of H-N-SN. ZC was prepared by incubating zymosan (ICN Pharmaceuticals, Inc., Cleveland, Ohio) with fresh rabbit or human serum, and washing extensively as previously described (25). ZC was stored frozen as aliquots at −70°C until used. ZC (3.75 mg) was incubated with 1×10^7/ml human neutrophils in Tyrode's-BSA at 37°C for 15 min with mixing. The reaction was stopped by centrifugation at 12,800 × G (Eppendorf 5412 centrifuge, Brinkmann Instruments, Inc., Westbury, N. Y.) and immediate removal of the supernatant. H-N-SN was used directly or stored at −20°C until used. Some supernatant was extracted before use, as described below.

Extraction of H-N-SN. The supernatant was extracted with a chloroform:methanol:water (6:6:5.4) system, previously reported by Pinckard *et al.* (26) to be optimal for PAF extraction. Methanol was added to H-N-SN in a ratio of 6:4, and after mixing at room temperature for 30 min, the precipitated BSA was removed by centrifugation at 3000 × G for 15 min. Water (1.4 parts) and chloroform (6 parts) were added to the supernatant with mixing, producing a biphasic system. The chloroform layer was removed by separatory funnel, and the chloroform was evaporated by using rotary evaporator under vacuum. Tyrode's-BSA was added to elute any activity from the glass tubes.

Thin-layer chromatography. H-N-SN and PAF were extracted into chloroform as described above and spotted on silica gel G plates (Analtech preparative plates, Fisher Scientific Co., Pittsburgh, Pa.). Thin-layer chromatography was run with a solvent system of chloroform:methanol:water (65:35:4) as described by Pinckard *et al.* (26). One-centimeter sections were scraped, suspended in chloroform:methanol:water (1:2:0.8) to elute activity, and silica gel G was removed by centrifugation. Chloroform and water were added to produce a ratio of chloroform:methanol:water 6:6:5.4, resulting in a biphasic system. To test for activity, the chloroform phase was evaporated, and activity was eluted into Tyrode's-BSA and tested with platelets as described below.

Rabbit platelet preparation. Rabbit platelets were incubated with ^{3}H-serotonin and prepared as previously described (5). They were used in Tyrode's-gelatin within 3 hr of preparation.

Human platelet preparation. Platelets were prepared over an erythrocyte pellet, by using modifications of a technique previously described by Bang *et al.* (27) and Benveniste *et al.* (9). Blood was taken into 1/7 volume acid citrate dextrose and centrifuged at 5900 × G for 60 sec to remove plasma. The pelleted components were resuspended in Tyrode's-gelatin with citrate and washed twice by using identical centrifugations. The pelleted cells were then resuspended in Tyrode's-gelatin without Ca^{++}, pH 7.2, and centrifuged at 275 × G for 10 min. The platelet suspension was removed and adjusted to 5×10^8/ml for aggregation experiments, with addition of bovine fibrinogen 1 mg/ml (Sigma) and $CaCl_2$ 4×10^{-3} M.

Platelet stimuli and inhibitors. Thrombin (bovine, Parke, Davis & Co., Detroit, Mich.) was diluted in Tyrode's-gelatin and stored as aliquots at −70°C. All other activators and inhibitors were prepared just before used. Adenosine diphosphate trisodium salt (Aldrich Chemical Co., Milwaukee, Wis.), hirudin (Sigma), phosphocreatine (CP; Sigma), and creatine phosphokinase (CPK; Sigma) were diluted in Tyrode's-gelatin. Arachidonic acid (Sigma) was dissolved in Tris-buffered saline, pH 8.6, with pH subsequently readjusted to 7.2 at final dilution. Indomethacin (Sigma) was dissolved to 10^{-3} M in ethanol, then to 10^{-5} M in Tyrode's-gelatin before addition. (Appropriate dilutions of ethanol alone were included as controls.) Eicosatetraenoic acid was kindly provided as a gift by Dr. W. Scott (Hoffman-LaRoche Labs) and dissolved in dimethyl sulfoxide to 10^{-3} M before final dilution in Tyrode's-gelatin. Rabbit PAF was made by incubation of horseradish peroxidase with 1×10^{-7} mononuclear cells containing approximately 6 to 10% basophils of appropriately sensitized rabbits as previously described (5). Rabbit neutrophil supernatant (R-N-SN) was prepared by incubating 1×10^7 rabbit neutrophils with ZC in a manner similar to that for H-N-SN production. Experiments with H-N-SN were performed by using unextracted material, and later were repeated with material extracted from the chloroform phase.

Release of serotonin. All reactions were performed in duplicate, by using methods previously described (5). The standard reaction mixture contained 2.5×10^8/ml platelets in 0.4 ml Tyrodes-gelatin. Reactions were performed in Eppendorf 1.5 ml centrifuge tubes, and were terminated by centrifugation at 12,800 × G. Supernatant (0.1 ml) was immediately removed and counted in a mixture of toluene and Aquasol (New England Nuclear, Pilot Chemicals Div., Watertown, Mass.) in a liquid scintillation counter. The secreted ^{3}H-serotonin was expressed as a percentage of the total determined by platelet lysis with Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.). Background secretion from platelets incubated without stimulus was subtracted and never exceeded 5% of total.

Platelet aggregation. The standard reaction mixture contained 2.5×10^8/ml platelets in 0.5 ml Tyrode's-gelatin. Reactions were performed at 37°C in a Payton Dual Channel Aggregometer (Payton Associates, Buffalo, N. Y.) with stirring at 1000 rpm with the use of techniques previously described (4).

Enzyme assays. Lactic dehydrogenase (LDH) (25), β-glucuronidase (β-gluc) (25), and myeloperoxidase (MPO) (23) were assayed by techniques previously described. Total enzyme val-

ues were measured after lysis with Triton X-100, and background values from cells incubated without stimulus were subtracted. Release of enzymes was expressed as a percentage of the total cellular enzyme activity from replicate samples.

RESULTS

Our recent work has demonstrated that rabbit neutrophils can be induced by incubation with ZC to release a material highly active for platelet stimulation. Current experiments confirm that the supernatant of human neutrophils, similarly stimulated with ZC, also contains a material active on rabbit platelets and with marked similarity to rabbit basophil-derived PAF.

The production of platelet-activating material from human neutrophils

Response of neutrophils to different concentrations of ZC. Different quantities of ZC, ranging from 0.25 to 7.5 mg, were added to 1×10^7 human neutrophils to determine the platelet-stimulating activity of the supernatant. As shown in Figure 1, with amounts as small as 0.5 mg, serotonin-releasing activity was detectable, and at 2.5 mg, ZC maximal activity was reached. No reduction in serotonin release was seen when higher doses of ZC were used. For experiments in which H-N-SN was further tested, the H-N-SN was generated by using 3.75 mg/ml ZC. It was also noted that in the absence of stimulation, neutrophils did not spontaneously generate similar platelet-activating material.

Kinetics of the ZC-neutrophil reaction. Platelet-activating activity, as measured by supernatant ability to effect serotonin release, was detectable shortly after addition of ZC to neutrophils. As seen in Figure 2*A*, activity first appeared at 5 min, and increased rapidly, reaching peak levels at 15 to 30 min. From this same experiment, release of several neutrophil enzymes was measured and is shown in Figure 2*B*. LDH was not released in significant quantities, indicating that the ZC-neutrophil reaction was not cytotoxic. The kinetics of the release of the neutrophil granular constituents, MPO, and β-gluc were somewhat different than the generation of platelet-secreting activity. Release of these enzymes was always seen to begin within the first 2 min of incubation, earlier than H-N-SN activity, and continued to rise through the 60-min incubation, long after serotonin-releasing activity reached a maximum.

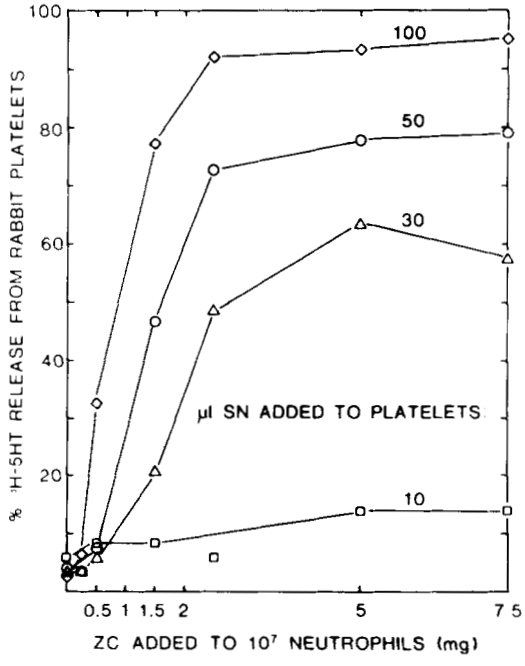

Figure 1. Dose response of ZC with neutrophils to generate serotonin-releasing activity in the supernatant. Increasing amounts of ZC were added to 1×10^7/ml neutrophils in Tyrode's BSA (37°C, 15 min). The reaction was stopped by centrifugation and supernatants were added to rabbit platelets. The curves represent different quantities of supernatants added.

The time course for neutrophil phagocytosis of ZC is depicted in Figure 2*C*. Phagocytosis proceeded rapidly and by 10 min, nearly all neutrophils contained ingested particles. (By this time, particles were so numerous that accurate counting was impossible.) Onset of phagocytosis was apparent by 1 min, and in all experiments in which it was studied, phagocytosis always preceded detection of serotonin-releasing activity.

Albumin and calcium requirements. The ZC-neutrophil reaction was ordinarily performed in buffer containing 0.25% BSA. When BSA was omitted or replaced with gelatin, serotonin-releasing activity was no longer detectable in the supernatant. These results are identical with those seen in the production of rabbit basophil-derived PAF and R-N-SN, where albumin has been shown to bind and stabilize activity (3, 7). Calcium was also shown to be required during ZC-neutrophil incubation in order for platelet-activating ability to be seen. When prepared neutrophils were initially washed in 1 mM EGTA before the addition of ZC, serotonin-releasing activity was only detectable when Ca^{++} was reintroduced at the time of incubation (results not shown).

Effect of preincubation of neutrophils with arachidonic acid pathway inhibitors on generation of platelet activity. To test the possibility that endoperoxides or thromboxane A_2 might be responsible for H-N-SN activity, neutrophil preparations were preincubated with indomethacin in concentrations that abrogated the platelet effect of arachidonic acid and that had been previously reported to lead to decreased leukocyte production of arachidonic metabolites (28). As depicted in Table I, these cells produced H-N-SN of equal potency to that produced by nontreated cells. Similarly, when the inhibitor eicosatetraenoic acid (ETYA) was preincubated with neutrophils, no change in activity was noted. After storage at −20°C, samples of these supernatants were subsequently assayed by Dr. J. Shaw at the University of Texas for presence of thromboxane B_2 by radioimmunoassay. No evidence of this metabolite was found in any sample to the lowest assay limit of 50 pg/ml, a concentration lower than that previously reported necessary to induce platelet activity (6, 29). In our samples, platelet-activating potential remained undiminished. The data indicate that these arachidonic acid metabolites are unlikely to be the active factor of H-N-SN (see also below).

Relationship of H-N-SN activity to percentage of eosinophils. Although neutrophils represented the great majority of cells present in the leukocyte preparations, eosinophils were always present, usually in low percentages. When cell preparations from different subjects, identical in total leukocyte concentration but varying in percentage of eosinophils, were incubated with ZC, the serotonin-releasing activity of the resultant supernatants exhibited no correlation to the number of eosinophils. Results of a typical experiment are shown in Table II. In other experiments, when cells from a single donor were prepared by differential centrifugations so that the neutrophil fraction varied in eosinophil content from 2 to 6%, no difference in supernatant ability to affect platelets was noted (results not shown).

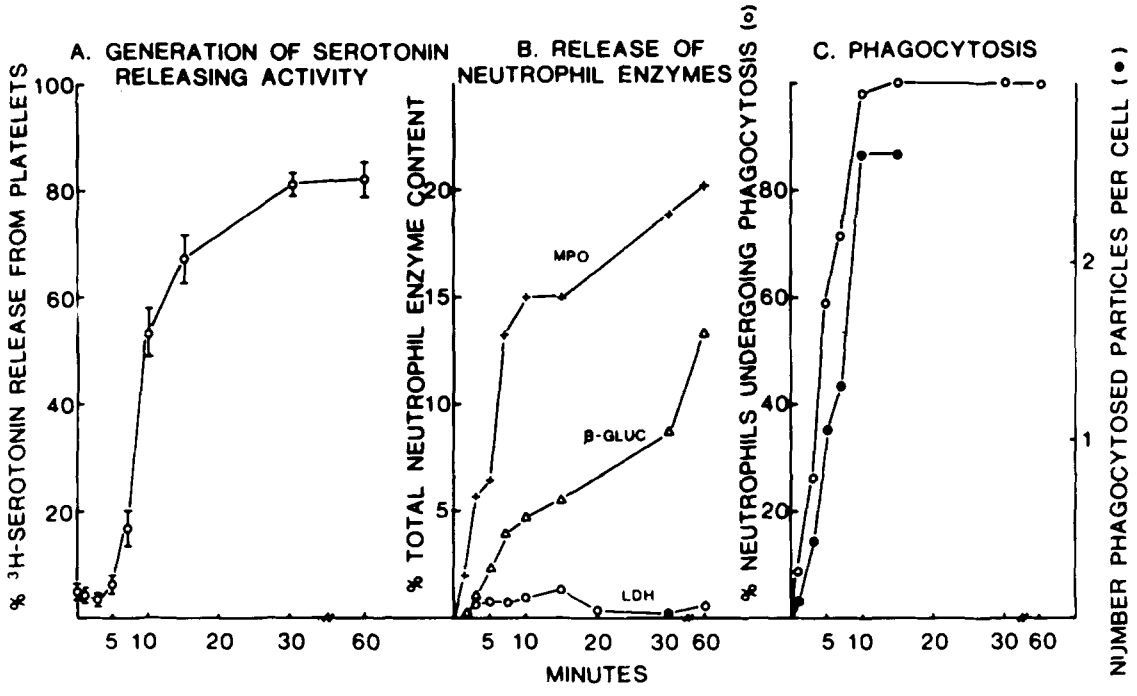

Figure 2. Time course of ZC-induced responses of neutrophils. ZC (3.75 mg) was incubated with 1×10^7 neutrophils at 37°C with stirring. *A*, production of platelet-activating activity, as measured by ability of supernatant to release ^{3}H-serotonin from rabbit platelets (means of replicate samples ± 1 S.E.M.); *B*, neutrophil enzyme release. MPO, β-gluc, and LDH are expressed as a percentage of total neutrophil enzyme content; *C*, phagocytosis of ZC particles. The reactions were stopped with 2% glutaraldehyde and smears were examined for percent of neutrophils showing ingested ZC particles (○—○), and number of particles/cell (●—●). In 10 min the latter was too high to accurately count.

TABLE I

Effect of preincubation of neutrophils with indomethacin or ETYA on generation of platelet-activating activity[a]

Concentration	^{3}H-5HT Release (±1 S.E.M.)
	%
Indomethacin	
None (buffer)	36.1 ± 2.5
5×10^{-5} M	38.5 ± 2.2
5×10^{-6} M	36.6 ± 6.9
5×10^{-7} M	38.5 ± 4.5
ETYA	
None (buffer)	42.0 ± 9.3
2×10^{-4} M	47.7 ± 8.6

[a] 1×10^7 neutrophils were preincubated with inhibitors for 120 sec before addition of ZC in triplicate samples. Fifteen microliters of the resultant supernatants were later added to 2.5×10^8 rabbit platelets to measure serotonin release.

TABLE II

Platelet activating activity related to percentage of eosinophils in neutrophil preparations

Preparation[a]	Eosinophils	^{3}H-5HT Release Rabbit Platelets (±1 S.E.M.)
	%	%
A	1.9	61.1 ± 1.1
B	2.0	38.5 ± 1.7
C	2.2	63.9 ± 3.4
D	2.9	45.5 ± 1.1
E	8.1	58.4 ± 2.9

[a] Neutrophil preparations from five normal subjects were adjusted to 1×10^7 cells/ml before incubating with ZC. Preparations were stained with Wright stain to determine the eosinophil percentage. Supernatants (10 μl) were added to rabbit platelets to measure serotonin-releasing activity. (The supernatants maintained the same rank order for platelet activation when added in higher or lower amounts.)

Effect of H-N-SN on rabbit platelets

The supernatant of human neutrophils incubated with ZC was reacted with rabbit platelets to determine its capacity for inducing aggregation and release.

Aggregation. Aggregation tracings from a typical experiment with graduated amounts of H-N-SN are seen in Figure 3. Several characteristics of the curves were apparent: 1) a shape change was noted without lag period; 2) the rate of aggregation was linear over a prolonged period; 3) there was a variable duration of aggregation; and 4) there was a rapid disaggregation at lower concentrations of the stimulus. Tracings of aggregation produced by the activators thrombin, PAF, and rabbit neutrophil supernatant are included in Figure 3 for comparison. The qualities of H-N-SN-induced aggregation are analogous to those previously described for antigen-induced rabbit PAF (4). Similar to that material and other platelet stimuli, measurements of the degree of aggregation (slope of the linear portion of curve, the height of the tracing, and the duration of aggregation) were directly related to the amount of H-N-SN added, whereas the rapidity of disaggregation was inversely related. Different preparations of H-N-SN, prepared from neutrophils of several donors and separate ZC preparations, induced aggregation curves of remarkable similarity.

Secretion of serotonin. Addition of H-N-SN to rabbit platelets in the presence of calcium could be readily demonstrated to induce ^{3}H-serotonin release. Figure 4 shows dose-response curves for this reaction. The response was similar with different preparations of H-N-SN, and led to the release of 80 to 98% of the total serotonin. Since the active material in H-N-SN is presently unknown, concentrations are expressed in terms of microliters of supernatant. ZC can be shown to have a direct effect on rabbit platelets; therefore control experiments were performed by incubating ZC in the absence of leukocytes. After

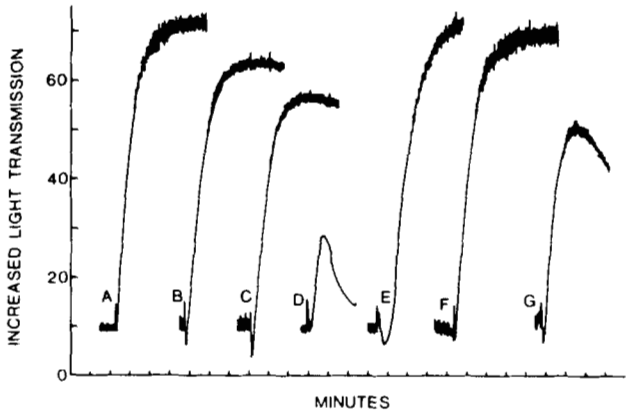

Figure 3. Tracings of H-N-SN-induced aggregations of rabbit platelets. Rabbit platelets at 2.5×10^8/ml in Tyrode's-gelatin were incubated at 37°C with stirring at 1000 rpm. Curves *A* to *D* show addition of H-N-SN 50 μl, 25 μl, 10 μl, and 2.5 μl. For comparison, other stimuli are shown in the remaining tracings: *E*, bovine thrombin 0.02 units; *F*, rabbit PAF 50 μl, and *G*, R-N-SN 50 μl.

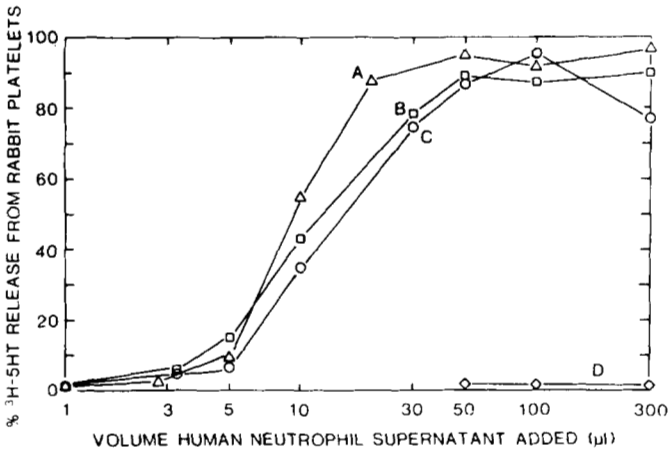

Figure 4. Dose response of H-N-SN-induced ^{3}H-serotonin secretion from rabbit platelets. Each curve represents the release from 2.5×10^8/ml rabbit platelets, incubated for 15 min at 37°C. Different preparations of H-N-SN were used in generating each curve. H-N-SN used in *A* and *B* were generated with zymosan opsonized in rabbit serum and in *C* with ZC in human serum. Curve *D* represents supernatant after ZC was incubated in the absence of neutrophils.

centrifugation, supernatants of these preparations failed to induce any serotonin release, indicating that the ZC was not responsible for the activity of H-N-SN (curve D). When assayed during these reactions, platelet LDH was never noted to be greater than 2% of total at any time point.

The kinetics of H-N-SN-induced secretion are demonstrated in Figure 5, having used different quantities of supernatant. The reaction proceeds rapidly, generally reaching completion at about 60 sec. This rapid response is again similar to that previously reported for release with PAF (5).

Comparison with known platelet activators. Experiments were conducted comparing H-N-SN with several known platelet activators to determine whether the activity in the supernatant was related to these substances. In the first experiment, 1 unit of thrombin was more than sufficient to cause maximal platelet aggregation and complete release of serotonin. These effects were competitively inhibited by 20 units of the leech extract, hirudin (Figure 6*A* and 7*A*). However, as may be seen, similar amounts of hirudin did not lessen either the aggregation or secretion-inducing effects of H-N-SN.

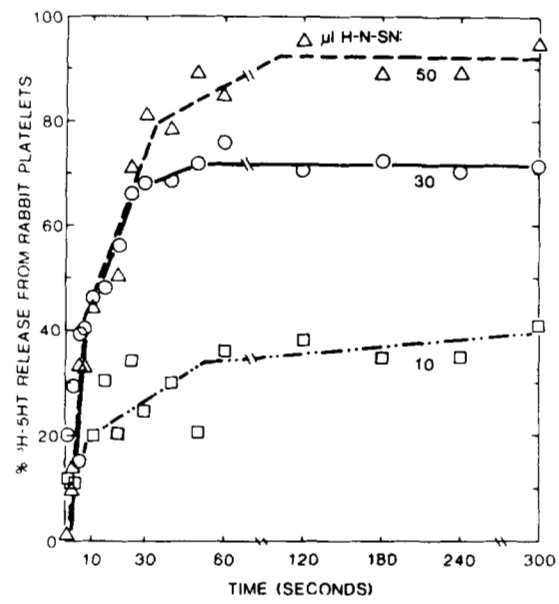

Figure 5. Kinetics of ^{3}H-serotonin secretion from rabbit platelets induced by different concentrations of H-N-SN added to 2.5×10^8 platelets.

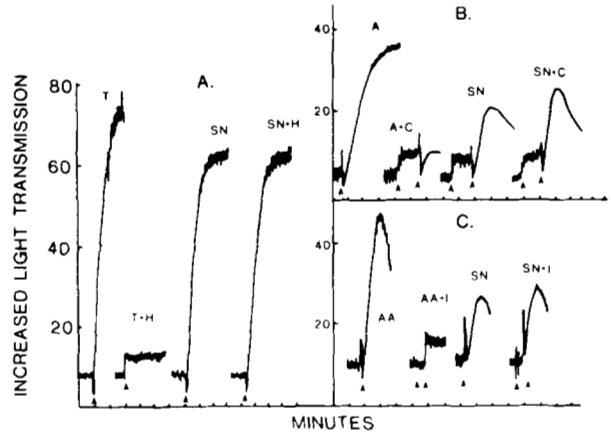

Figure 6. Comparison of H-N-SN to other stimulants of rabbit platelet aggregation. *A*, the aggregation induced by 1 unit of thrombin (T) was inhibited when platelets were preincubated for 60 sec with 20 units hirudin (H); H-N-SN (SN) activity was unaffected. *B*, aggregation induced by 1×10^{-5} M ADP (A) was inhibited by preincubation with 2 mM CP and 4 units/ml CPK (C); H-N-SN-induced aggregation was not diminished. *C*, the effect of 4×10^{-5} M arachidonic acid (AA) was abrogated by preincubation with 1×10^{-7} M indomethacin (I), (H-N-SN activity remained unchanged).

Similarly, the effects of ADP on platelet aggregation and secretion were abolished by incubation of platelets with CP and CPK. As shown in Figures 6*B* and 7*B*, 1×10^{-5} M ADP caused aggregation and weak serotonin release; the addition of the CP/CPK system markedly diminished these responses. When H-N-SN was used in quantities that gave similar aggregation responses, CP/CPK inhibited neither aggregation nor serotonin release.

The effects of arachidonic acid on platelets are mediated through products of its metabolism by the cyclo-oxygenase pathway. Thus, the aggregation and serotonin release induced by 4×10^{-6} M arachidonic acid were abolished by the cyclo-oxygenase inhibitor, indomethacin at a concentration of 1×10^{-7} M (Figs. 6*C* and 7*C*). As can be seen, responses to H-N-SN were not diminished by the addition of indomethacin.

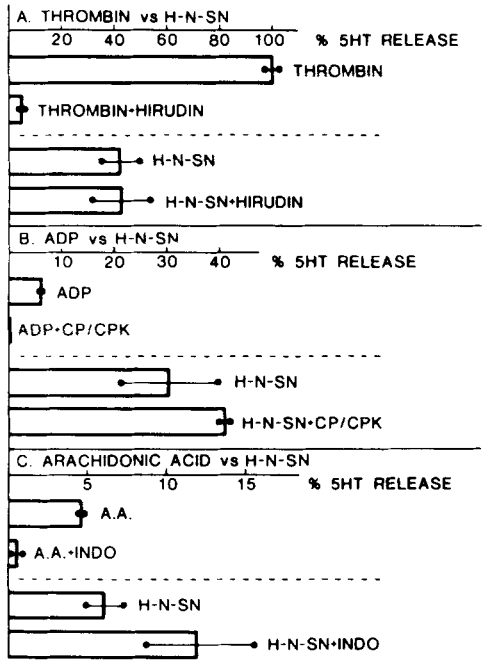

Figure 7. Comparison of H-N-SN to other stimulants of rabbit platelet ^{3}H-serotonin secretion. Activators and inhibitors were used at same concentrations as described in Figure 4. All incubations were done in triplicate. *A*, thrombin and hirudin; *B*, CP and CPK and ADP; *C*, indomethacin and arachidonic acid. Bars represent mean of replicates ±1 S.E.M.

Extraction into chloroform:methanol:water. Chloroform and methanol were used to extract the supernatant (aqueous) in a ratio of 6:6:5.4, and the chloroform layer was removed and dried by rotary vacuum evaporation. The contents were then resuspended in 0.25% BSA buffer and could be shown to possess both aggregating and serotonin-releasing activity similar to the original supernatant material (Fig. 8). When dilutional factors were considered, the potency of the original H-N-SN and the chloroform-extracted layer were almost identical. In contrast, the methanol-water layer, when processed in an identical manner, could be shown to possess only weak activity in inducing either platelet function.

Thin-layer chromatography. H-N-SN and rabbit PAF were compared by separation on silica gel G plates using a solvent system of chloroform:methanol:water (65:35:4). After 1-cm fractions were scraped and extracted from the silica gel into Tyrode's-BSA, the activity of each fraction was tested for the ability to activate rabbit platelets. As seen in Figure 9, the mobility patterns for H-N-SN and PAF were seen to be virtually identical; the Rf of 0.11 calculated for each material was similar to the previously published value of 0.10 in this system for PAF (26). H-N-SN and PAF have also been studied on several other chromatographic systems by Clark *et al.* (18). Their studies thus far have failed to distinguish between the two materials, both of which are easily separable with these methods from radiolabeled thromboxane B_2 and PGG_2.

Effects of H-N-SN on human platelets

Human platelets, prepared by an erythrocyte-pelleting technique, could be readily shown to aggregate in the presence of human neutrophil supernatant. Typical tracings are shown in Figure 10, in which platelets from four donors were prepared simultaneously by using identical techniques and incubated with 100 μl of H-N-SN. The individual variation of the aggregations is striking and is much greater than that which is usually observed when rabbit platelets are studied. In curve B, a biphasic aggregation is seen, a pattern that usually indicates secondary response to release of endogenous ADP. (This secondary reaction was abrogated by preincubation with CP/

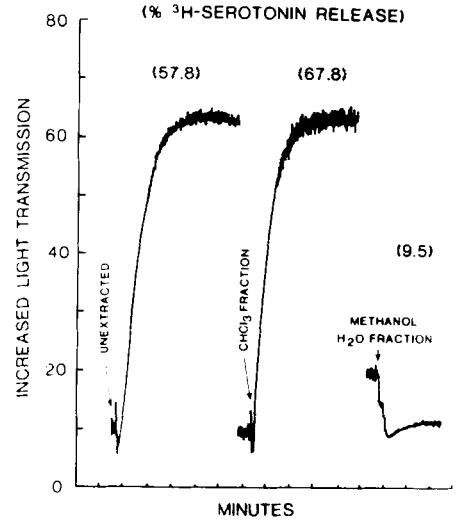

Figure 8. Extraction of H-N-SN activity into chloroform:methanol: water. H-N-SN was extracted as described in *Materials and Methods.* The two phases, chloroform and methanol:water, were each evaporated and resuspended in 5 ml Tyrode's-BSA. The aggregation tracings were produced by addition to tritiated rabbit platelets of 50 μl of the original H-N-SN, the chloroform-extracted fraction and the methanol:water-extracted fraction. At 1 min, an aliquot of the reaction mixture was removed, centrifuged, and assayed for ^{3}H-serotonin release as indicated above the curves.

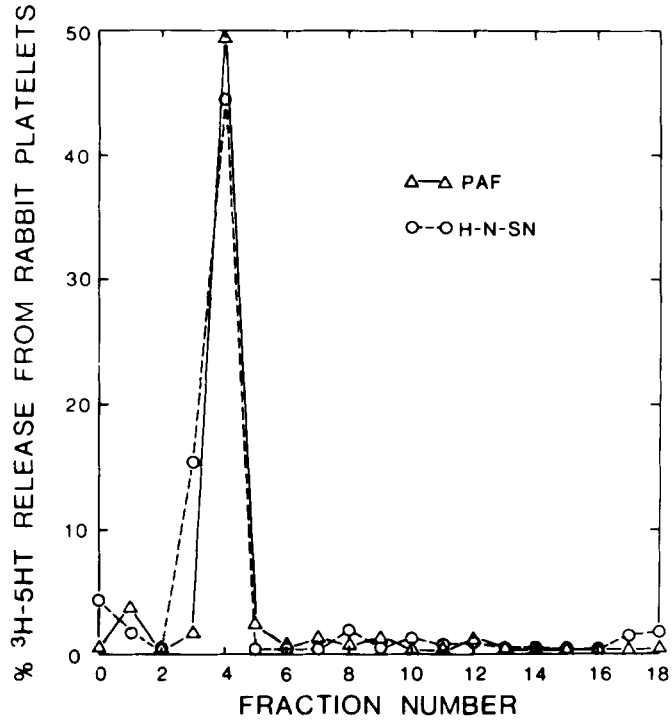

Figure 9. Thin layer chromatography of H-N-SN and PAF. As described in the text, H-N-SN and PAF (in chloroform) were spotted on silica gel G plates and run in chloroform:methanol:water 65:35:4. The origin was at the 2 cm mark. Eluates from 1-cm fractions were tested for platelet activity by their ability to induce ^{3}H-serotonin release from rabbit platelets.

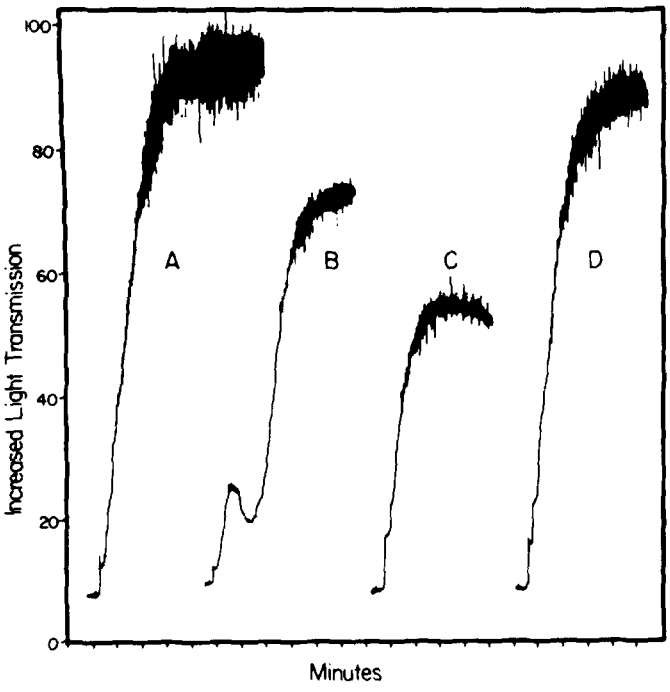

Figure 10. Tracings of H-N-SN-induced aggregations of human platelets. Platelets from four different donors were identically prepared by erythrocyte-pelleting technique to a concentration of 5×10^8/ml, and reacted with H-N-SN 100 μl. Individual variation is noted. The biphasic nature of curve *B* represents a secondary response to release of endogenous ADP.

CPK.) This behavior also is different from the response of rabbit platelets to H-N-SN.

Although all human platelets prepared by erythrocyte-pelleting aggregated in response to H-N-SN, release of serotonin was not as consistent. In some experiments, release of as much as 20% ^{3}H-serotonin release was observed; in others, platelet preparations that demonstrated maximal aggregation failed to show any serotonin release whatsoever. This variability, the potentiating effect of ADP, and the requirement to date for this particular method of human platelet preparation are currently under investigation. Human platelets prepared as described were also responsive to the material that had been eluted from thin-layer chromatography.

DISCUSSION

The present studies demonstrate that a material derived from human neutrophils exhibits potent effects on rabbit platelets. Our observations confirm earlier reports that a PAF-like material can be isolated in man (7, 16, 19–21) and demonstrates that such a factor can be produced from basophil-free cell preparations.

Activation of rabbit platelets. H-N-SN was seen to be a potent activator of rabbit platelets, for both aggregation and ^{3}H-serotonin secretion. Aggregation proceeded rapidly, after an immediate shape change, and unless doses had been large, disaggregation followed promptly. Serotonin release was also rapid and vigorous. In a reaction almost complete within 1 min, larger quantities of H-N-SN induced 90 to 98% serotonin release. The release reaction was seen to be noncytotoxic, as analysis of platelet enzymes showed that liberation of LDH did not occur (results not shown).

To further characterize the nature of the factor responsible for these reactions, we compared H-N-SN to several known pletelet stimulators and were able to show different patterns of activity and inhibition. H-N-SN activity was unaffected by the leech extract, hirudin, whereas hirudin was able to completely inhibit the effect of thrombin in doses otherwise sufficient to effect maximal aggregation of total serotonin release. These experiments rule out thrombin as the potential active factor of H-N-SN.

It is also unlikely that ADP is the active factor in H-N-SN or serves as a major component of the pathway through which the supernatant is active. ADP, even in quantities far exceeding usual physiologic concentrations, was never able to generate rabbit platelet responses of similar intensity to that seen with H-N-SN. Also, ADP did not require the presence of albumin to maintain activity and is not extracted into chloroform. In addition, the H-N-SN activity was unaffected by the presence of CP and CPK, a system that abrogated the ADP effects on platelets. As noted earlier, however, ADP may have a potentiating role in the response of human platelets to H-N-SN.

Arachidonic acid can cause both platelet aggregation and serotonin release (30). However the effect of arachidonic acid was abolished by preincubation of the platelet with the cyclo-oxygenase inhibitor, indomethacin, whereas H-N-SN activity was not diminished. This indicates that H-N-SN activity is not operating through the intact arachidonic acid biometabolic pathways of the platelet. In addition to arachidonic acid itself, several products of this pathway, including the endoperoxides PGG_2 and PGH_2, and their unstable derivative thromboxane A_2, also produce potent effects on platelets (30–32). Recently, Goldstein *et al.* (28) have shown that human neutrophils can generate thromboxane A_2 in response to phagocytic stimuli. However, the production of H-N-SN from neutrophils treated with indomethacin (a cyclo-oxygenase inhibitor) or ETYA (an inhibitor of both cyclo-oxygenase and lipoxygenase), the stability of H-N-SN, and the demonstration of platelet activity in the absence of detectable thromboxane B_2 suggest that thromboxane A_2 is not responsible for H-N-SN activity. This conclusion is further strengthened by the observations of Clark *et al.* (18) showing that H-N-SN active factor is chromatographically separable from arachidonic acid, PGG_2, and thromboxane B_2.

Activation of human platelets. As seen in Figure 10, H-N-SN readily induces aggregation of human platelets prepared by pelleting over erythrocytes. Platelets prepared by several other techniques (centrifugation analogous to the rabbit platelet preparation; gel filtration as described by Fine *et al.*(33); albumin-density gradient as described by Walsh *et al.* (34)) were not seen to be as responsive. With these methods, H-N-SN induced a shape change and occasionally very minor aggregation. Although it is not unusual for human platelets prepared by different methods to show differential sensitivities to a particular stimulus, the *in vitro* response to H-N-SN demands much further investigation. Other characteristics of the human platelet response, including poor reproducibility of serotonin release and possible synergistic responses with ADP indicated by several experiments, distinguish the human system from the rabbit sufficiently as to exclude extrapolating any generalizations from the rabbit to man. The nature of the response of human platelets to H-N-SN is currently under investigation.

Relationship to PAF. The properties of rabbit basophil-derived PAF have been thoroughly studied for several years, but its chemical structure has not yet been established. Thus, it is not presently possible to demonstrate a structural identity between H-N-SN and PAF. Nevertheless, several lines of evidence demonstrate a remarkable similarity and suggest PAF and H-N-SN may be the same substance. 1) Both platelet

activators required the presence of albumin for stabilization. 2) The characteristic parameters of H-N-SN induced aggregation of rabbit platelets appeared identical to those previously described for PAF-induced aggregation. 3) The kinetics of rabbit platelet serotonin secretion induced by H-N-SN were virtually identical to those previously established for release effected by PAF, with rapid onset and completion near 1 min. In addition, pletelets aggregate to lower concentrations of either material than is required to effect release, a distinction that is not seen with several other platelet activators (5). 4) When the chloroform:methanol:water extraction system previously shown to be optimal for PAF extraction was employed (26), H-N-SN activity was seen almost entirely in the chloroform phase, a pattern identical to that observed when PAF was similarly extracted. This does not necessarily indicate that H-N-SN is identical to PAF, but does indicate a lipid nature for each. 5) The identical migration of rabbit basophil-derived PAF and H-N-SN on several thin-layer chromatographic systems and identical response to chemical treatment (18) constitute the strongest evidence for the similarity of the two materials. In summary, by the physical, chemical, and functional criteria currently employed, we have been unable thus far to show any differences between rabbit PAF and human neutrophil supernatant.

Cellular origin of H-N-SN. Previous reports have indicated that human material with PAF-like properties has originated from basophils (20, 21). In man, basophils constitute a much smaller fraction of the total leukocytes than in the rabbit. Thus, Lewis *et al.* (20) used a patient with basophilic leukemia as the source of a markedly enriched, although potentially abnormal, basophil preparation to demonstrate that platelet activity could be generated in the presence of the calcium conophore A23187. Camussi *et al.* (21) used a buffy coat preparation in their experiments, in which the basophils could be shown to degranulate in response to the stimuli used. However, the cell populations were quite heterogeneous. Thus, to date, the evidence for the basophil as the cell of origin of possible PAF in man remains controversial.

The cell preparations in the preparations described herein have been essentially basophil-free. As previously noted, counting of 1000 leukocytes usually revealed no basophils, and were always <0.2% of total cells. This evidence neither confirms nor denies the earlier evidence for human PAF deriving from basophils; it does strongly indicate that another human cell type is capable of generating PAF-like material.

The overwhelming majority of cells present in our preparations were neutrophils. Eosinophils were always observed, generally ranging from 1 to 4.6% in nonatopic donors, and to 10% in atopic donors. Experiments were conducted incubating ZC with neutrophil preparations from different donors, identical in total cell concentration but varying in eosinophils from 1.4 to 8.1%. In other studies (data not shown), by differential centrifugation, the neutrophil fraction of a single donor could be varied to contain 2 to 6% eosinophils. In both cases, the ability of the resulting supernatants to activate platelets exhibited no correlation to the percentage of eosinophils present. It has been shown that preparations of rabbit peritoneal neutrophils, which are devoid of eosinophils, release as much, if not more, platelet-activating activity than peripheral neutrophil preparations, when stimulated by zymosan (ZC). Although these results do not exclude the eosinophil as a source of H-N-SN, they strongly suggest that the material described herein derives from neutrophils. Preliminary experiments suggest that a mononuclear cell fraction, depleted of neutrophils, can also generate platelet-activating factor when stimulated by ZC. Further investigation will be necessary to demonstrate which other human cell types besides the neutrophil are able to produce this material.

The origin of a human platelet-activating material from neutrophils (and possibly other inflammatory cells) opens many areas for speculation. Certainly, there is a possibility that PAF may prove to be a much broader mediator of inflammation than previously realized. The presence of platelets in inflammatory reactions has often been noted in the past, but the role of the platelet has been poorly understood. However, further work will be necessary to define the effects of H-N-SN on human platelets or other cells *in vivo*, the conditions necessary for its release, and its potential role in inflammatory or hypersensitivity reactions.

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