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Platelet activating factor: an inhibitor of neutrophil activation?

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SUMMARY

Preparations of platelet activating factor (PAF) derived by methanolic extraction of supernatants from antigen-challenged rabbit basophils proved capable of activating platelets while concurrently inhibiting neutrophil aggregation/secretion stimulated by biologically active F-Met peptides, ionophore A23187, or zymosan-treated serum. This inhibition was non-cytotoxic and species-non-specific. When these PAF preparations were analysed using thin-layer chromatography, multiple lipids were detected. Both platelet-stimulating as well as neutrophil-inhibitory activity was present in a lipid component migrating at an R_F consistent with native PAF; however, these biological activities were not limited only to PAF and, indeed, could also be detected in lipid with solubility characteristics more closely related to a lysophosphatide than to native PAF. These data are compatible with the belief that native PAF may belong to a family of biologically active lipids differing somewhat in physico-chemical properties. Moreover, these data illustrate that PAF and/or PAF-like molecules may also demonstrate a biological activity distinct from their effects upon the platelet.

INTRODUCTION

Platelet activating factor (PAF), a neutral, low molecular weight polar lipid originally isolated from the supernates of sensitized rabbit basophils undergoing antigenic challenge, binds to and activates both rabbit and human platelets. *In vitro*, PAF induces the rapid aggregation of platelets and the secretion of vasoactive amines (Henson, 1970; Benveniste, Henson & Cochrane, 1972; Benveniste, Le Couedic & Kamoun, 1975; Henson, 1976; O'Donnell, Henson & Fiedel, 1978) predominantly independent of the action of released ADP or the generation of biologically active metabolites of arachidonate (Henson, 1977; Shaw *et al.*, 1978; Cazenave, Benveniste & Mustard, 1979). *In vivo*, a role for PAF has been implicated in models of serum sickness (Henson & Cochrane, 1971; Camussi, Mencia-Huerta & Benveniste, 1977), the Arthus reaction (Kravis & Henson, 1977) and IgE-mediated systemic anaphylaxis (Pinckard *et al.*, 1977); additionally, a PAF-like activity has been described in a number of patients with thrombotic thrombocytopenic purpura (Lian *et al.*, 1979). Recently, a material chemically indistinguishable from rabbit basophil PAF has been reported to be released from activated rabbit (Lynch *et al.*, 1979) and human (Lotner *et al.*, 1980) neutrophils; this, and the previously reported binding of rabbit basophil PAF to rabbit neutrophils (Shaw & Henson, 1976) as well as the demonstration of neutrophils in PAF-mediated platelet aggregates (Benveniste *et al.*, 1972; Pinckard *et al.*, 1977), suggested that PAF might be involved in multiple cellular

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interactions. In view of these observations we have investigated the effects of basophil-derived PAF upon the neutrophil. We report herein that PAF derived by methanolic extraction of supernatants from antigen-challenged rabbit basophils was capable of activating platelets while concurrently inhibiting neutrophil aggregation and secretion. When these supernatants were analysed by thin-layer chromatography, multiple lipid components were identified; both platelet-stimulating and neutrophil-inhibitory capacity were present in lipid migrating at an R_F value consistent with native PAF. However, these activities were not limited only to native PAF. These data suggest that PAF may possess biological activity separable from its effect upon the platelet and further, that PAF-like activity may well be associated with other normally occurring lipids physico-chemically similar to PAF and thus representing a family of closely related molecules.

MATERIALS AND METHODS

Solutions. Solutions used for the preparation of PAF were prepared as described (Henson, 1976): isotonic phosphate-buffered saline (PBS), Tris–Tyrode's solution (without Ca^{++} or Mg^{++}) in the presence and absence of ethylene glycol tetra-acetate (EGTA), Tyrode's bovine serum albumin (BSA) and Tyrode's gelatin. All solutions were adjusted to pH 7·2. Hanks' balanced salt solution (HBSS; GIBCO, Grand Island, New York) with or without added Ca^{++}/Mg^{++} (10 mM $CaCl_2$/3·5 mM $MgCl_2$) was used in the isolation of neutrophils; bovine (0·01%; Sigma Chemical Company, St Louis, Missouri) or human (1·0%; Worthington Biochemical Corp., Freehold, New Jersey) serum albumin was included in the HBSS for use with rabbit or human neutrophils respectively.

Immunization. New Zealand white (NZW) rabbits were immunized subcutaneously with horseradish peroxidase (1 mg/ml in saline; HRP type II, Sigma Chemical Company) (Henson, 1976), reimmunized with 5 mg/ml HRP at intervals of 1–3 months, and bled 10 days post secondary immunization via cardiac puncture.

Preparation of standard PAF. Crude preparations of PAF were isolated by the methanolic extraction of supernates from antigenically challenged rabbit basophils, as described (Henson, 1976; O'Donnell *et al.*, 1978); these supernates, termed standard PAF, were stored at −70°C. Prior to use, the methanolic PAF was blown to dryness and the lipid residue dissolved in 0·15 N NaCl with BSA (2·0 mg/ml). Neither sham-challenged HRP-sensitized leucocytes nor HRP-challenged normal leucocytes released PAF activity.

Thin-layer chromatography. PAF was purified further by thin-layer chromatography on silica gel G-plates (500 μm; Applied Science Laboratories, State College, Pennsylvania) according to Shaw *et al.* (1978). Standard PAF was applied to the plates which were subsequently developed in chloroform:methanol:water (65:35:4, v/v); the plates were then air-dried, sectioned and scraped. A reference plate was sprayed with molybdenum blue (Dittmer & Lester, 1964) and/or exposed to iodine vapour to locate lipid standards which included lysolecithin (LL), phosphatidyl choline (L), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS) and sphingomyelin (SM) (Applied Sciences); arachidonic acid was obtained from Nu Chek Prep. Inc., Elysian, Minnesota.

Samples scraped from the plates were added to chloroform:methanol:water (1:2:0·8, v/v) and agitated by use of a vortex mixer. After elution at 22°C for 10 min, the samples were centrifuged (1,000 *g* for 10 min), the clear solvent layer was removed, dried down, resuspended in the 0·15 N NaCl-BSA diluent, and assayed for an ability to induce platelet secretion and/or alter neutrophil aggregation; these samples are termed PAF-TLC. In certain experiments PAF-TLC was isolated using a more polar solvent system consisting of chloroform:methanol:water at 65:35:6, v/v.

Preparation of neutrophils. Rabbit peritoneal cells were obtained from NZW rabbits that had been challenged at 14-day intervals with 0·1% glycogen in saline (Cohn & Morse, 1959). Rabbits were injected intraperitoneally with 100 ml of glycogen–saline and 4 hr later 100 ml of heparinized saline (10 u/ml) was introduced into the peritoneal cavity. The cell exudate was withdrawn by gravity drainage as the abdomen was gently kneaded. The exudate fluid was centrifuged at 150 *g* for 8 min at 4°C to sediment the peritoneal cells, hypotonic lysis was performed on the cell pellet and the remaining granulocytes ($\geq$90% neutrophils) were washed and resuspended to 10^7 cells/ml in HBSS

(without Ca^{++} and Mg^{++}) containing 0·01% BSA (O'Flaherty *et al.*, 1977). After a 15-min equilibration on ice, the cell suspension was incubated with cytochalasin B (CB; Aldrich Chemical Co. Inc., Milwaukee, Wisconsin) at 5 μg/ml for 15 min at 37°C.

Human peripheral blood neutrophils were prepared as described by Böyum (1968). Blood from a normal donor was directed into heparinized vacutainer tubes and layered on Ficoll–Hypaque. After centrifugation at 400 *g* for 20 min at 22°C, the granulocyte layers were mixed with 5% dextran in normal saline, contaminating erythrocytes were removed, hypotonic lysis was performed (× 3) and the granulocytes were then washed in HBSS–HSA (without Ca^{++} and Mg^{++}) and resuspended in the same buffer to 10^7 cells/ml. The cells were allowed to equilibrate on ice for 40 min before incubation with CB at 5 μg/ml (15 min at 37°C) (Craddock, White & Jacob, 1978). The cells obtained represented 95% neutrophils.

Stimuli. The synthetic peptides *N*-formyl-L-methionyl-L-phenylalanine (F-Met-Phe) and *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (F-Met-Leu-Phe) were obtained from Andrulis Research Corp. (Bethesda, Maryland); peptides were dissolved at 10^{-2} M in dimethylsulphoxide (DMSO) and diluted to 10^{-6} M in buffer for use. The ionophore A23187 (a generous gift from Dr Robert Hamill of Eli Lilly Research Laboratories, Indianapolis, Indiana) was dissolved in DMSO at 5 mg/ml (10^{-2} M) and diluted to 10^{-4} M in sterile water. Zymosan A particles (Sigma Chemical Co.) were boiled in 0·15 N NaCl for 60 min, washed twice, resuspended to 4 mg/ml in normal saline, and frozen at −70°C until use. Zymosan-treated serum (ZTS) was prepared by incubating washed zymosan with autologous serum at 4 mg/ml for 30 min at 37°C with shaking. Zymosan particles were removed from the serum by centrifugation and the ZTS was diluted 1 : 7 into the experimental tubes.

Granulocyte aggregometry. Standard platelet aggregometry techniques were followed according to Craddock *et al.* (1977) using a dual-channel aggregometer/recorder system (Payton Associates Inc., Buffalo, New York). Granulocyte aggregation was assessed by adding 0·1 ml of HBSS (with Ca^{++} and Mg^{++}) to 0·5 ml of the cell suspension in a siliconized cuvette. After a 3-min incubation at 600 r.p.m. varying amounts of buffer, standard PAF or PAF-TLC were added as changes in light transmission were continually recorded; cells were activated 1 min later with the F-Met peptides, A23187, or ZTS and recording continued for an additional 3 min. To provide the necessary amplification for recording a well-defined aggregation wave, the aggregometer/recorder system was calibrated to use a 4 mV range with a fresh granulocyte suspension diluted 70% (v/v) in buffer serving as the 100% aggregation end-point.

Enzyme assays. Lactic dehydrogenase (LDH) was assayed according to a modification of the methods described by Wroblewski & LaDue (1955); β-glucuronidase was measured as described by Brittinger *et al.* (1968). Total enzyme levels were obtained from the supernatants of cells subjected to cycles of freeze-thawing.

RESULTS

Effect of standard PAF upon the aggregation of rabbit neutrophils

The addition of the F-Met peptides or ionophore A23187 to rabbit peritoneal neutrophils resulted in progressive and irreversible increases in light transmission and oscillation amplitude (Fig. 1) when measured by aggregometry; admixture of an amount of standard rabbit PAF, which induced 50% of the maximal inducible release of ^{14}C-serotonin from platelets (O'Donnell *et al.*, 1978), with neutrophil suspensions prior to challenge with the peptides or ionophore greatly reduced the aggregation response normally observed. Inhibition of neutrophil aggregation was proportional to the amount of standard PAF incorporated in the aggregometry system such that the doubling of the PAF (Fig. 1) resulted in the complete abrogation of aggregation induced by either stimuli. In simultaneous experiments, β-glucuronidase release was depressed to an extent roughly equivalent to the decrease in aggregation observed, indicating that standard rabbit PAF was inhibiting a generalized activation state; this inhibition was non-cytotoxic with $< 10\%$ release of LDH in the presence of PAF. Standard PAF incubated alone with rabbit neutrophils did not stimulate their aggregation or the secretion of β-glucuronidase nor was it chemotactic when assessed using a

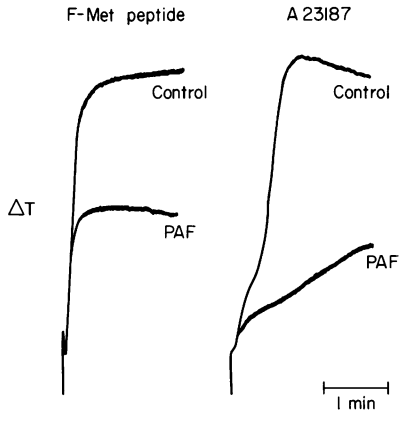

Fig. 1

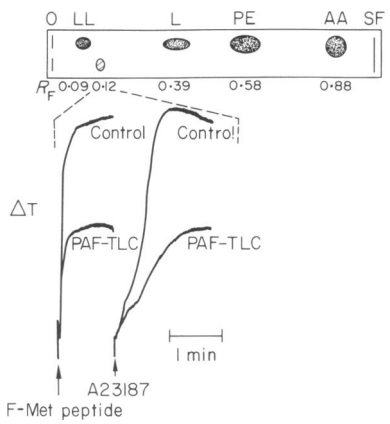

Fig. 2

Fig. 1. Representative aggregation profiles of rabbit neutrophils induced by F-Met peptides or A23187 and their inhibition by standard rabbit PAF. The PAF concentration used was an amount which produced the 50% maximal inducible secretion of ^{14}C-serotonin from platelets. Doubling of the PAF concentration completely abrogated aggregation to either stimulus. ΔT = change in relative light transmission.

Fig. 2. Schematic of a thin-layer chromatogram of standard rabbit PAF (PAF-TLC; lower tract) and reference lipids (upper tract) developed in $CHCl_3$:MeOH:H_2O (65:35:4, v/v) and the inhibition by PAF-TLC of rabbit neutrophil aggregation stimulated by F-Met peptides or A23187; inhibition was observed with a singular lipid component migrating at an $R_F = 0{\cdot}12$. PAF-TLC concentration was standardized as described for standard PAF in Fig. 1. O = origin; LL = lysolecithin; L = lecithin; PF = phosphatidyl ethanolamine; AA = arachidonic acid; SF = solvent front. ΔT = change in relative light transmission.

Boyden chamber chemotaxis radioassay (Gallin, Clark & Kimball, 1973); further, random migration as measured in these chambers remained unaffected in the presence of standard PAF. These data suggested that standard PAF contained at least two biologically discernible activities: stimulation of the platelet and inhibition of the neutrophil. It remained unclear whether these activities resided in the same molecule, hence further purification was attempted using thin-layer chromatographic (TLC) techniques.

Effect of PAF-TLC fractions upon the aggregation of rabbit neutrophils

In TLC, as depicted in Fig. 2, a lipid component migrating with an $R_F = 0{\cdot}12$, and previously characterized as native rabbit PAF (Pinckard, Farr & Hanahan, 1979), duplicated results obtained with standard PAF in the rabbit neutrophil aggregation assay; this material also contained the platelet-activating properties previously described (O'Donnell *et al.*, 1978). Various other lipid components were also detected in standard PAF; all were essentially without effect in the platelet/-neutrophil assay systems. The relatively close R_F values for native PAF (0·12) and the LL reference lipid (0·09) indicated some similarity between the two molecules; indeed, should LL be present in standard PAF preparations, its complete separation from PAF using this solvent system would not be certain.

When standard PAF was developed in a somewhat more polar solvent (chloroform:methanol:H_2O, 65:35:6, v/v), a major lipid was detected migrating at an $R_F = 0{\cdot}26$, in agreement with Pinckard *et al.* (1979) for native PAF in this system. Once again, various lipids were separated during thin-layer chromatography of standard PAF, including one migrating at an R_F (0·20) similar to that of authentic LL (0·19) (Table 1). Native PAF, lipid migrating at $R_F = 0{\cdot}20$ and authentic LL all non-cytotoxically inhibited neutrophil aggregation (Table 1); moreover, the $R_F = 0{\cdot}20$ component, like native PAF, non-cytotoxically stimulated ^{14}C-serotonin secretion from both rabbit and human platelets. By contrast, LL (at sub-lytic levels) did not itself stimulate amine secretion from

Table 1. Relative mobility, inhibition of neutrophil aggregation, and platelet secretion of 5HT: comparison of reference lipid and a representative PAF isolate*

Compound	R_F	Maximal inhibition in neutrophil aggregation (%)†	Secretion of platelet 5HT (%)‡
Reference Lipid			
PE	0·70	n.d.	n.d.
PS	0·51	n.d.	n.d.
L	0·46	n.d.	n.d.
SM	0·36	n.d.	n.d.
LL	0·19	30–100	⩽2
Standard PAF			
	Origin	⩽3	⩽2
	0·12	⩽3	⩽2
	0·20	40–60	21–29
	0·26§	40–60	22–33
	0·36	⩽3	⩽2

* Chloroform:methanol:H_2O, 65:35:6, v/v.

† Activation measured by aggregometry using F-Met peptides and A23187 as stimulating agents. Inhibition presented as % maximal decrease in extent of aggregation as described in Materials and Methods. LL was used at 20–110 μM; PAF chromatographic isolates were brought to volume in sufficient buffer for duplicate assays.

‡ Per cent ^{14}C-5HT secreted; LL was used to 500 μM with PAF isolates prepared as described.

§ Native PAF.

n.d. = Not determined.

platelets. Solvent extraction of the area between the $R_F = 0{\cdot}20$ component and native PAF (Table 1) did not result in the isolation of any biologically active component(s) when tested in either the platelet or neutrophil assay system, reflecting the absence of unvisualized material of high biological activity overlapping both the R_F 0·20 and 0·26 spots.

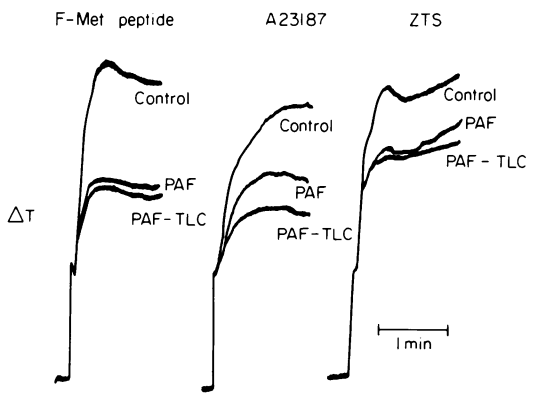

Fig. 3. Cross-species activity of rabbit basophil-derived PAF. Illustrated are representative aggregation profiles of human neutrophils induced by F-Met peptides, A23187, or by zymosan-treated serum (ZTS), and their inhibition by both standard rabbit PAF and PAF-TLC (concentrations chosen as described in Figs 1 and 2). ΔT = change in relative light transmission.

Effect of standard rabbit PAF and PAF-TLC on human neutrophil activation

Rabbit basophil-derived PAF activates human platelets (O'Donnell *et al.*, 1978); this cross-species capability was also demonstrated with human neutrophils. As depicted in Fig. 3, both standard PAF of rabbit origin or the major PAF-TLC fractions ($R_F = 0{\cdot}12$ and $0{\cdot}26$ respectively) inhibited human neutrophil aggregation and secretion of β-glucuronidase following stimulation with either active peptides, ionophore, or ZTS. Inhibition was, as in the homologous system (Fig. 1), non-cytotoxic as shown by $\leqslant 10\%$ release of LDH.

DISCUSSION

For a number of years, there has existed a controversy concerning the chemical nature of PAF. Our previous suggestion (O'Donnell *et al.*, 1978) that a family of related molecules with PAF-like activity might exist was based, at least in part, upon a report (Kravis & Henson, 1975) that PAF liberated from rabbit lung tissue had properties similar to basophil-derived PAF but was not identical since platelets desensitized to the basophil material still responded to lung PAF. Furthermore, the differential sensitivities of PAF prepared in various laboratories to the enzymatic action of phospholipases tended to support the concept of a family of PAF-like molecules (Kater, Austin & Goetzel, 1975; Benveniste *et al.*, 1977; Pinckard *et al.*, 1979). Recently, a glyceryl ether phospholipid was shown to be biologically indistinguishable from native PAF, and concurrently, an acetylated LL was shown to exhibit PAF-like activity but to be 200-fold less active than the glyceryl ether (Demopoulos, Pinckard & Hanahan, 1979). Moreover, PAF has been reported to be an alkyl ether analogue of LL (Benveniste *et al.*, 1979).

In the present study, we provide evidence for the existence of two distinct PAF-like activities in the challenge supernatants of specifically immunized rabbit basophils. One of these is a lipid component consistent with native PAF (Pinckard *et al.*, 1979) and the other more physico-chemically related to the lysophosphatide LL by virtue of its mobility characteristics in TLC. However, this PAF-like molecule, which migrates in the chloroform:methanol:H_2O (65:35:6, v/v) developing system at an $R_F = 0{\cdot}20$, behaves biologically more like native PAF than LL. Although native PAF, $R_F = 0{\cdot}20$ lipid and LL all depress neutrophil aggregation (and secretion of β-glucuronidase) following stimulation with a variety of agents, only PAF and $R_F = 0{\cdot}20$ lipid were able to activate the platelet. Indeed, LL (at sub-lytic concentrations) failed to stimulate the platelet to aggregation or secretion of 5HT in agreement with previous reports (Joist *et al.*, 1977; Fiedel, 1978).

We believe this description of two distinct molecules with PAF-like activity may in part address the inability of numerous investigators to agree upon uniform biological and physico-chemical properties for PAF. More important, however, is the observation that platelet activating factor(s) may have a biological ability clearly separate from the platelet, i.e. the non-cytotoxic inhibition of neutrophil aggregation and enzyme secretion stimulated by a variety of inducing agents. This speaks to a potential role for PAF in both the immune and non-immune neutrophil infiltration that occurs during acute inflammatory vascular damage as well as the recently demonstrated (Jacob *et al.*, 1980) intravascular leucoembolization important in the clinical manifestations of myocardial infarction, shock lung and pulmonary dysfunction. Exactly what role is played by PAF, of course, remains to be determined.

Lastly, claims have been made for the presence of PAF or a PAF-like activity in cell types other than the basophil/mast cell, including the platelet (Chignard *et al.*, 1979), the monocyte (Clark, Hanahan & Pinckard, 1979) and the neutrophil (Lynch *et al.*, 1979; Lotner *et al.*, 1980). It will be important to isolate and compare the molecules responsible for these PAF-like activities to assess truly whether PAF is as ubiquitous as presently might seem or an activity associated with a variety of closely related molecules dispersed throughout a spectrum of cell types.

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