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# PHYSICAL AND CHEMICAL PROPERTIES OF PLATELET-ACTIVATING FACTOR OBTAINED FROM HUMAN NEUTROPHILS AND MONOCYTES AND RABBIT NEUTROPHILS AND BASOPHILS

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## Summary

Platelet-activating factor secreted by stimulated human neutrophils and monocytes and rabbit neutrophils and basophils was isolated, purified and chemically and physically characterized. All four preparations had identical thin layer chromatographic behavior in a variety of solvent systems, identical solubility behavior in various organic and aqueous solvents and responded comparably to a variety of chemical and physical agents designed to reveal the presence functional groups. These findings indicate that human neutrophil and monocyte, and rabbit neutrophil and basophil derived platelet-activating factor preparations are indistinguishable, and support the conclusion that platelet-activating factor is a neutral, polar lipid molecule whose functional activity is dependent upon the presence of a carboxylic acid ester.

## Introduction

Platelet-activating factor, a soluble, lipid-like platelet stimulator has been reported to be released from a variety of cells from several mammalian species in response to several stimuli that include antigen-stimulated, IgE-sensitized rabbit [1] and human [2] basophils and rat peritoneal cells [3,4]; rabbit and human mast cells and basophils stimulated with antigen-IgG immune complexes [5]; human neutrophils [6], human monocytes and rabbit neutrophils stimulated with opsonized zymosan (Henson, P.M., personal communication); and porcine buffy coat leukocytes [7]. The factor is a most potent stimulator of rabbit platelets both in vivo and in vitro, inducing a calcium-independent shape change, and calcium-dependent aggregation and secretion of platelet granular constituents, including histamine, serotonin, and platelet factor 4

[1,8—14]. The rabbit basophil-derived factor has a solubility and thin layer chromatographic behavior characteristic of a neutral, polar lipid compound [14].

This study provides evidence for the establishment of the comparable physicochemical nature of the factor obtainable from four cell sources: human monocytes, human neutrophils, rabbit neutrophils, and rabbit basophils, following stimulation of these cells either with opsonized zymosan [6] or specific antigen [14—16]. The identification of the factor from these inflammatory cells suggests the involvement of platelets in a variety of inflammatory reactions and acute vascular tissue injury.

## Materials and Methods

*Experimental animals.* Male and female randomly bred Californian rabbits were obtained from Penn Acres Ranch, Wimberly, TX.

*Platelet-activating factor production from rabbit basophils.* Immunization procedures for the production of antibody only of the IgE class, as well as procedures for the quantitation of IgE antibody production have been described previously [15,16]. The preparation of buffy coat leukocytes containing IgE sensitized basophils and their in vitro antigenic stimulation to release the basophil-derived factor as well as the extraction and subsequent purification of the resultant factor have been recently reported [14—16].

*Platelet preparation.* Washed, [$^3$H]serotonin-labeled rabbit platelets were prepared by a recent modification [14] of the method originally described by Ardlie et al. [17].

*Quantitation of platelet-activating factor activity.* Samples (4—20 $\mu$l) of the factor dissolved in bovine serum albumin-saline (2.5 mg/ml) were added to 200 $\mu$l of washed, prewarmed (37°C), [$^3$H]serotonin-labeled platelets ($2.5 \times 10^8$ cells/ml in Tyrodes solution, pH 7.2) in plastic 12 × 75 mm test tubes. The suspensions were immediately mixed, and incubated at 37°C for 1 min. The release reaction was halted by the addition 20 $\mu$l ice-cold 1.5 M formaldehyde solution, together with immediate cooling to 0°C. The tubes were centrifuged at 2200 × *g* for 20 min at 4°C to sediment platelets, and 25 $\mu$l supernatant was removed to quantitate the release of [$^3$H]serotonin. The percentage of serotonin release was determined relative to that released by the addition of 4 $\mu$l 2.5% Triton X-100 to the same volume of platelets, under identical conditions. Secretion profiles were plotted as percent of serotonin release versus volume of the factor added. Such profiles routinely demonstrated a linear response of serotonin secretion to factor addition (activity) over a secretion range of 5—40%.

*Thin layer chromatography.* Precoated Silica gel G thin layer plates (Analtech, Newark, DE) were divided into individual lanes and activated for at least 3 h at 105°C prior to use. After application of the factor samples and phospholipid standards, the plates were dried for an additional 5 min at 105°C, and placed in solvent tanks containing the desired solvent system. The chromatograms were developed at room temperature until the solvent front had migrated approx. 15 cm above the origin, the lanes containing the factor were sectioned into 0.5-cm fractions starting at the origin, and were removed from the plate.

The factor was extracted from each silica gel fraction with $CHCl_3/CH_3OH/H_2O$ (1:2:0.8, v/v) as previously described [14]. The factor activity was identified by the stimulation of [$^3$H]serotonin secretion from washed rabbit platelets. The lanes containing the phospholipid standards were then sprayed with phosphate-detecting reagent [19] or were sprayed with sulfuric acid and heated to 600°C for detection of the reference phospholipids.

*Solubility behavior of platelet-activating factor.* The behavior of the factor in a variety of organic and aqueous solvents was determined in the following manner: samples of the factor in $CHCl_3$ (10—50 μl) were dried in glass reaction tubes (12 × 75 cm) at 30°C under $N_2$ followed by the addition of 1 ml the respective solvent. The tubes were exposed to solvent for 30 min at room temperature with periodic mixing. The solvent was then removed and transferred to a second, glass transfer tube. Both reaction and transfer tubes were dried under $N_2$ and the factor activity was quantitated as discussed above and compared to non-treated control samples of the factor.

*Physicochemical treatments.* Dried factor samples in glass test tubes were placed in a drying oven and incubated at 110°C for 40 min. Base catalyzed methanolysis was accomplished in methanolic (0.5 N) NaOH with incubation at room temperature for 2 min, followed by acidification of the sample (addition of a slight excess of 3 N HCl). The reaction mixture was made biphasic, $CHCl_3/CH_3OH/H_2O$ (1:1:0.9, v/v) and the $CHCl_3$-rich layer neutralized by removing the upper $CH_3OH/H_2O$ layer and washing repeatedly until a neutral pH was established. Treatment with aqueous base was done by the addition of aqueous NaOH solutions ranging from 0.05 to 1.0 N to dried platelet-activating factor samples at room temperature for up to 20 min. The reactions were terminated by the addition of a slight excess of HCl. The samples were then extracted with 60% aq. $CH_3OH$, made biphasic, and washed to neutrality as described above. Acid treatment of the factor was accomplished in a two-phase system. Samples were prepared in $CHCl_3/CH_3OH$ (9:1, v/v) to which were added 0.5 vols. 3 N HCl. The samples were incubated at room temperature for 20 min with mixing. The $CH_3OH/H_2O$ layer then was removed and the chloroform layer washed to neutrality as described above. PAF dissolved in $CHCl_3/CH_3OH$ (9:1, v/v) was reacted with 0.5 vol. 28% $NH_4OH$ for 20 min at room temperature with mixing, followed by the addition of a slight excess of HCl. Periodic acid treatment was performed by mixing equal volumes of the factor in $CH_3OH$ with 0.1 M methanolic $HIO_4$ at room temperature for 2 h in the dark. The samples were then made biphasic and washed to neutrality as described above. Upon completion of each of the above procedures the factor (phased into $CHCl_3$) was dried under a nitrogen stream and quantitated for residual activity relative to sham controls by stimulation of platelet secretion.

*Chemicals.* All solvents and chemicals were reagent grade and were used without further purification or treatment. Pre-coated thin layer chromatography plates (Silica gel G, 250 μm and 1000 μm thicknesses) were obtained from Analtech, Newark, DE. Phosphatidyl choline was prepared according to Bajwa and Hanahan [20]; sphingomyelin was purified from bovine erythrocytes [21]; and lysolecithin was prepared by the action of phospholipase $A_2$ (from *Crotalus adamanteus* venom, Miami serpentarium) on phosphatidylcholine in diethyl ether, followed by purification on thin layer chromatography [22].

## Results

*Thin layer chromatography (TLC)*

Platelet-activating factor obtained from rabbit neutrophils and human monocytes and neutrophils was partially purified by TLC. Preparative (1000 μm thickness) chromatograms containing acetone-washed rabbit neutrophil, and human monocyte and neutrophil-derived factor were developed in $CHCl_3$/$CH_3OH$/$H_2O$ (65:35:4, v/v) simultaneously with a highly purified rabbit basophil-derived factor [14] serving as a reference marker, as well as three standard phospholipids, phosphatidylcholine, sphingomyelin, and lysolecithin. All of the PAF samples migrated identically under these conditions to a point similar to the lysolecithin standard (Table I). Subsequently, analytical TLC (250 μm thickness) in solvent systems with different pH values was employed to examine certain physical properties of the factor molecule. The $R_F$ of all four factor samples remained unchanged during chromatography in neutral ($CHCl_3$/$CH_3OH$/$H_2O$, 65:35:4, v/v),alkaline ($CHCl_3$/$CH_3OH$/$NH_4OH$, 65:35:4, v/v), and acidic ($CHCl_3$/$CH_3OH$/$CH_3COOH$/$H_2O$, 25:15:4:2, v/v) systems (Table I) yet the mobilities of the reference phospholipids were altered by these changes in solvent pH, indicating that the factor is structurally dissimilar to any of these three lipids.

Platelet-activating factor mobility was, however, significantly altered by a change in the polarity of the developing solvent. Table I reveals that an increase in solvent polarity ($CHCl_3$/$CH_3OH$/$H_2O$, 65:35:6, v/v) resulted in a significant increase in PAF mobility relative to that of the standard phospholipids. These findings particularly emphasize the likely structural differences between the factor and the standard phospholipids, and exemplify the similarity between the factor activities obtained from these four cell types.

TABLE I

MIGRATION OF PLATELET-ACTIVATING FACTOR DURING TLC

Samples of each factor were placed at the origin of thin layer plates. The solvent front was allowed to migrate approx. 15 cm, and the plates marked into fractions for location of factor activity. The $R_F$ of the factor could then be calculated from the active factor fractions. $R_F$ values for standard lipids could be calculated after reaction with phosphate reagent [19]. Standard lipids: LL, lysolecithin; S, sphingomyelin; PC, phosphatidylcholine. Factor source: RB, rabbit basophils; RN, rabbit neutrophils; HN, human neutrophils; HM, human monocytes.

| Solvent | $R_F$ * | | | | | | |
|---|---|---|---|---|---|---|---|
| | Standard lipids | | | Factor source | | | |
| | LL | S | PC | RB | RN | HN | HM |
| $CHCl_3$/$CH_3OH$/$H_2O$ (63:35:4, v/v) | 0.09 | 0.20 | 0.31 | 0.09 | 0.09 | 0.09 | 0.09 |
| $CHCl_3$/$CH_3OH$/$NH_4OH$ (63:35:4, v/v) | 0.13 | 0.19 | 0.31 | 0.09 | 0.09 | 0.10 | 0.10 |
| $CHCl_3$/$CH_3OH$/$CH_3COOH$/$H_2O$ (25:15:4:2, v/v) | 0.09 | 0.14 | 0.24 | 0.10 | 0.09 | 0.09 | 0.09 |
| $CHCl_3$/$CH_3OH$/$H_2O$ (63:35:6, v/v) | 0.14 | 0.29 | 0.46 | 0.25 | 0.25 | 0.25 | 0.25 |

* $R_F$ = relative mobility = distance travelled by sample/distance travelled by solvent front.

*Solubility studies*

A variety of organic solvents, as well as several aqueous solvent systems, were employed to assess the solubility behavior of the factor. The results, shown in Table II, illustrate that the factor was soluble only in the more polar organic solvents, and in aqueous systems only in the presence of albumin (2.5 mg/ml).

*Physicochemical studies*

Evidence for the presence or absence of certain functional groupings in the active factor molecule was assessed through a series of physical and chemical treatments of the factor. Since the amount of factor available for investigation was extremely small, all of these tests were based upon the recovery of functional factor activity only, and were intended to uncover any source-related differences among the four factor sources tested. The most informative result was obtained by the base-catalyzed methanolysis of the factor, which after treatment for as little as two minutes or less at room temperature resulted in the complete destruction of functional activity. In a similar manner the factor activity was susceptible to aqueous base (in the absence of $CH_3OH$), as demonstrated by the complete loss of all factor activity after 20 min in 0.5 N NaOH (aq.) at room temperature.

Platelet-activating factor was found to be acid resistant, as evidenced by its resistance to 3 N HCl for up to 20 min, as well as its resistance to the phosphate-detecting reagent applied to TLC plates, from which highly active factor fractions could be extracted. The factor was resistant to periodic acid treatment (0.1 M in $CH_3OH$ for 2 h), to weak bases (28% $NH_4OH$), and to heat (110°C, 40 min) in the presence of atmospheric oxygen.

TABLE II

SOLUBILITY CHARACTERISTICS OF PLATELET-ACTIVATING FACTOR

Aliquots of each factor from soluble stocks ($CHCl_3/CH_3OH/H_2O$, 1:2:0.8, v/v) were treated with solvents and the factor activity determined in the reaction and transfer tubes, then compared to the initial factor activity (non-treated controls).

| Solvent | % Factor activity (HM, HN, RN, RB) | | |
|---|---|---|---|
| | Initial | Reaction tube | Transfer tube |
| Diethyl ether (dry) | 100 | 100 | 0 |
| Diethyl ether (water satd.) | 100 | 100 | 0 |
| $CHCl_3$ (dry) | 100 | 100 | 0 |
| $CHCl_3$ (water satd.) | 100 | 100 | 0 |
| Acetone | 100 | 93 | 7 |
| Tyrode's solution [23] | 100 | 100 | 0 |
| $CH_3OH$ | 100 | 0 | 100 |
| $C_2H_5OH$ | 100 | 35 | 65 |
| $CHCl_3/CH_3OH/H_2O$ (1:2:0.8, v/v) | 100 | 0 | 100 |
| $CHCl_3$ * ($CH_3OH/H_2O$ satd.) | 100 | 0 | 100 |
| Tyrode's/albumin ** (2.5 mg/ml) | 100 | 0 | 100 |
| Albumin **/saline | 100 | 0 | 100 |

* $CHCl_3$-rich layer taken from $CHCl_3/CH_3OH/H_2O$ (1:1:0.9, v/v).

** Bovine serum albumin.

## Discussion

In the course of the present investigations we have examined platelet-activating factor obtained from four separate cell sources from two species (human monocytes and neutrophils, and rabbit neutrophils and basophils) and have obtained substantial physical and chemical evidence that these factor molecules either are structurally identical, or display only very minor structural differences. The behavior of the factor during thin layer chromatography indicates that even though the factor is a lipid, it differs structurally from three conventional types of phospholipids (phosphatidylcholine, sphingomyelin, and lysolecithin). This conclusion is supported by the fact that the $R_F$ of the factor remains unchanged in response to changes in solvent pH, while those of the reference lipids change significantly (Table I). This behavior suggests that the factor either is a neutral molecule with no ionizable groups, or that it possesses charged groups that are not altered by changes in solvent pH, or that any changes in charged groups offset one another such that the net charge on the factor molecule remains unchanged. The resistance of the factor to treatment with a weak base (28% $NH_4OH$) argues additionally that the factor is a neutral molecule.

That the factor is a relatively polar lipid molecule is evident from both its thin layer chromatographic behavior and its solubility in various organic and aqueous solvents. In one chromatographic environment ($CHCl_3/CH_3OH/H_2O$, 65:35:4, v/v) the factor displays a low $R_F$ (0.09, Table I) migrating coincidentally with lysolecithin, while in a solvent of higher polarity ($CHCl_3/CH_3OH/H_2O$, 65:35:6, v/v) its $R_F$ increases dramatically, (0.25, Table I) migrating coincidentally with sphingomyelin, positively demonstrating its polar nature while eliminating lysolecithin and sphingomyelin from consideration as the factor. Solubility of the factor only in the relatively polar organic solvents such as methanol, ethanol, and single-phase mixtures containing chloroform, methanol, and water, together with its insolubility in nonpolar organic solvents (chloroform, and diethyl ether) and aqueous systems in the absence of albumin, further supports its relatively polar lipid composition (Table II).

Even though a definite chemical structure for the factor from any source has not yet been reported, it is possible, based on its reactivity in various simple chemical tests, to compare the factor from these four cell sources with respect to the presence or absence of specific chemical groupings. The most significant observation made in this regard is the rapid and complete destruction of factor activity exposed to methanolic NaOH. Susceptibility to base-catalyzed methanolysis argues that the factor contains one or more acyl functions that are critical to its functional activity. These would most likely be in the form of carboxylate esters naturally found in most glycerides as well as other lipid classes. In a similar manner the susceptibility of the factor to aqueous as well as alcoholic NaOH provides further evidence for the presence of critical acyl functions in the active molecule. Resistance to high temperature in the presence of atmospheric oxygen suggests that the aliphatic side chains of these fatty acids are either saturated or that any unsaturated regions are not important to the expression of functional factor activity.

Finally, the factor is resistant to treatment with periodic acid in methanol, and aqueous HCl, suggesting that the functionally active molecule contains

neither vicinal hydroxyls, hydroxy-amines, nor vinyl ether groupings. It should be re-emphasized that throughout these physical and chemical investigations, the factor from all of the cell sources could not be differentiated in their responses to any of the experimental agents, indicating that these molecules are in all likelihood one and the same with respect to both functional activity and molecular structure.

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## Addendum

Since the initial submission of this manuscript we have synthesized a glyceryl ether-containing lipid (1-*O*-alkyl-2-acetyl-*sn*-glycerol-3-phosphorylcholine) with physicochemical properties and biological activity indistinguishable from that of native, rabbit basophil-derived factor [Demopoulos, C.A., Pinckard, R.N., and Hanahan, D.J. (1979) J. Biol. Chem., in press.]. The presence of the acetyl moiety is essential for the biological activity of rabbit basophil and neutrophil, and human monocyte and neutrophil-derived factors, as well as the synthetic glyceryl ether lipid. Thus, quantitative recovery of the biological activity of hydrolyzed ($CH_3OH$/NaOH) factor samples is accomplished by reaction of these samples with acetic anhydride (30 min, 60° C), providing further evidence for the structural similarity if not identity among the platelet-activating factors from these four sources and the acetyl glyceryl ether lipid.

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