Metabolic, Membrane, and Functional Responses of Human Polymorphonuclear Leukocytes to Platelet-Activating Factor

By Leah M. Ingraham, Thomas D. Coates, John M. Allen, Coleen P. Higgins, Robert L. Baehner, and Laurence A. Boxer

The phospholipid mediator of anaphylaxis, platelet-activating factor (PAF) is chemotactic for polymorphonuclear leukocytes (PMN). We have examined this agent's effects on several other PMN functions. Human PMN were prepared from heparinized venous blood by Ficoll gradient. Metabolic burst was examined by measurement of O₂ use and O₂ production in the presence or absence of PAF (10⁻¹⁰-10⁻⁶ M). Unless cells were treated with cytochalasin-B (5 μg/ml), no significant respiratory burst was demonstrated. However, pretreatment with PAF (10⁻⁷ M) enhanced approximately threefold the O₂ utilization found when cells were subsequently stimulated with 10⁻⁷ M FMLP. PAF also stimulated arachidonic acid metabolism in ¹⁴C-arachidonic acid-labeled PMN. Thin-layer chromatography analysis of chloroform-methanol extracts showed substances that comigrated with authentic 5-hydroxyeicosatetraenoic acid had a marked increase in radioactivity following PAF stimulation at 10⁻⁷ M. PAF failed to stimulate release of granule enzymes, B-glucuronidase, lysozyme, or myeloperoxidase unless cytochalasin-B were added. PAF from 10⁻⁴ M to 10⁻⁴ M affected PMN surface responses. PMN labeled with the fluorescent dye, chlorotetracycline, showed decreased fluorescence upon addition of PAF, suggesting translocation of membrane-bound cations. Further, the rate of migration of PMN in an electric field was decreased following PAF exposure, a change consistent with reduced cell surface charge. PMN self-aggregation and adhesion to endothelial cells were both influenced by PAF (10⁻⁶ M-10⁻³ M). Aggregation was markedly stimulated by the compound, and the percent PMN adhering to endothelial cell monolayers increased almost twofold in the presence of 10⁻³ M PAF. Thus, PAF promotes a variety of PMN responses: enhances respiratory burst, stimulates arachidonic acid turnover, alters cell membrane cation content and surface charge, and promotes PMN self-aggregation as well as adherence to endothelial cells.

Much attention has been directed in the past several years to the effects of the synthetic chemotactic peptides on function of polymorphonuclear leukocytes (PMN). N-formyl-methionyl-leucyl-phenylalanine (FMLP), in addition to its chemotactic effects, promotes a variety of responses from PMN including metabolic burst, degranulation, and aggregation. Recently, the phospholipid mediator of anaphylaxis, platelet-activating factor (PAF), has been shown to be chemotactic for PMN. This compound is 1-0-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (AGEPC), and it is released from leukocytes of a variety of species including human, rabbit, and rat. The in vivo effects of PAF, of either physiologic or synthetic origin, are the same and include hypotension, neutropenia, and thrombocytopenia. On the cellular level, PAF promotes aggregation of platelets and chemotaxis in neutrophils. We wished to determine if, like FMLP, PAF has other possible regulatory effects on PMN function. We report the results of our investigation of PAF influence on PMN metabolism, membrane and cell surface changes, and the functional responses of degranulation, aggregation, and adherence.

Materials and Methods

Chemicals

Synthetic PAF, designated l-0-lecithin-β-acetyl-y-O-alkyl, was obtained from Calbiochem-Behring Corp. (La Jolla, Calif.). The preparation that was >98% pure was that of Do and Ramachandran from chicken egg yolk. The alkyl groups are predominantly hexadecyl and octadecyl species. A stock solution (2.5 x 10⁻⁴ M) was prepared by dissolving 1 mg in 7.49 ml of methanol:chloroform (3:1). This solution was stored at –20°C. Shortly before use, a sample of the stock was withdrawn and dried under nitrogen gas. A solution of 0.9% NaCl with 0.25% bovine serum albumin (BSA) (Fraction V, Sigma Chemical Co., St. Louis, Mo.) was added to give a concentration of 10⁻⁴ and 10⁻¹ M. Chlorotetracycline HCl (CTC), cytochrome-C, type VI, bovine serum albumin (BSA), superoxide dismutase (SOD), FMLP, cytochalasin-B, and enzyme substrates were obtained from Sigma Chemical Co. (St. Louis, Mo.). Authentic 5-hydroxyeicosatetraenoic acid (5-HETE) was supplied by Dr. M. Siegel of Burroughs Wellcome Co. (Research Triangle Park, N.C.). ¹⁴C-arachidonic acid (55.8 μCi/μmole) was obtained from New England Nuclear (Boston Mass.). Shortly before use, the isotope was dried under N₂ gas, and converted to the sodium salt by addition of 0.1 M sodium carbonate. Powdered medium 199 (with Earle's salts, without NaHCO₃), l-glutamine, neomycin sulfate solution, and fungizone (amphotericin B) were purchased from Gibco Laboratories, Grand Island biological Co., Grand Island, N.Y.

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Supported in part by Grant RO1 AI 10892-08 and RO1 AI-16948-01 from the National Institutes of Health and a grant from the Riley Memorial Association. This work was done during the tenure of L.A.B. as an Established Investigator of the American Heart Association.

Submitted July 1, 1981; accepted January 28, 1982.

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Cell Preparation

Heparinized venous blood was obtained from normal volunteers in accordance with the precepts established by the Helsinki Declaration. PMN were separated by Ficoll gradient and freed of contaminating erythrocytes by hypotonic lysis. Final suspension of cells (95%-99% PMN) was in phosphate-buffered saline (PBS) or Kreb's Ringer phosphate (KRP). In some instances, 5.5 mM glucose was added to the suspending medium (PBSG or KRP).

Oxygen Consumption

The oxygen consumption by PMN was measured by use of an oxygen electrode (Model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio) with an expanded scale Varian recorder (Vaian Associates, Palo Alto, Calif.). A suspension of 10⁷ cells in KRP was placed in the reaction vessel. After 5 min of incubation at 37°C, Na azide (final concentration 1 mM) and other agents to be tested were added to give a final volume of 3 ml. Baseline oxygen consumption was measured for 5 min and then 100 µl stimulant was added. Oxygen consumption was recorded an additional 5–12 min. Results of replicate samples were averaged and expressed as nmoles O₂ used/5 min/10⁷ cells.

Superoxide Production

Superoxide ion was measured as the superoxide dismutase (SOD) sensitive reduction of cytochrome-C as described by Babior et al. Each tube had in a final volume of 0.9 ml: 0.75 ml of KRP containing 3×10⁷ cells, 119 µM cytochrome-C, and 1 mM sodium azide. In some instances, SOD (60 µg) and/or cytochalasin-B (5 µg) were added. After 5 min equilibration at 37°C, the reaction was initiated by addition of 100 µl stimulant. At the end of the reaction time (1, 2, 5, or 10 min), the tube was placed in ice and 0.5 ml of ice-cold 1 mM N-ethylmaleimide (NEM) was added. The tubes were centrifuged (400 g, for 5 min, 4°C) and the supernatant absorptions were read at 550 nm on a Gilford Spectrophotometer (Model 250, Gilford Instruments Co., Oberlin, Ohio). The results of replicate samples were averaged and converted to nanomoles reduced cytochrome-C by use of the extinction coefficient of 19.1×10⁴ M⁻¹ cm⁻¹.

Arachidonic Conversions

PMN were suspended in PBSG with 1% BSA, fatty acid free (PBSGB), at a concentration of 10⁷ cells/ml. 4°C-sodium arachidonate (1–2 Ci/2×10⁵ cells) was added. The cells were incubated in a shaking water bath at 37°C for 30 min. Ten volumes of PBSGB were added and the cells were pelleted by centrifugation (150 g, 7 min, 20°C). Cells were resuspended in PBSG to give a cell density of 10⁷ cell/ml, and additions of stock solutions of sodium chloride and magnesium sulfate were added to give final concentration of 1.2 M of each of the divalent cations. After 5 min incubation in a shaking water bath at 37°C, stimulating agent was added and the incubation continued 5 additional minutes. Ice-cold citric acid (final concentration 15 mM) was added to stop the reaction. The acidified suspension was extracted with chloroform and methanol by a modified Bligh and Dyer extraction. The chloroform phase was dried under nitrogen and redissolved in 100 µl volume of chloroform:methanol (2:1, v/v). Forty-five-microliter samples of the extracts were spotted on silica gel G plates (Uniplate, Analtech, Newark, Del.) for analysis by thin-layer chromatography. The plates were developed in one of two solvents: (1) chloroform:methanol:glacial acetic acid (90:8:1.08, v/v/v) or (2) hexane:ethyl ethyl ether:glacial acetic acid (50:50:1, v/v/v). Rₜ values for phosphatidylcholine, PGE₂, 5-HETE, and arachidonic acid are, respectively, for solvent 1: 0.02, 0.4, 0.57, and 0.76; and for solvent 2: 0.01, 0.03, 0.28, and 0.65. Location of standards was visualized in iodine vapor, and the lanes for each sample were scraped in 0.5-cm sections. The radioactivity associated with sections corresponding to authentic 5-HETE was determined in a Packard Model 460D Liquid scintillation spectrometer (Packard Instruments, Downey, Calif.). Results of replicate samples were averaged and expressed as dpm/10⁷ cells.

Fluorescence Measurements

All fluorescence measurements were made in a Perkin-Elmer MPF-44B fluorescent spectrophotometer (Perkin-Elmer Corp. Instrument Div., Norwalk, Conn.) equipped with a temperature-controlled cuvette holder and automatic stirrer. CTC (final concentration 10 µM), was added to the cell suspension (3×10⁷ PMN/ml in Hepes balanced salt containing 10 mM Hepes, pH 7.35, with 130 mM NaCl, 4.5 mM KCl, and 5.5 mM glucose). CaCl₂ (1.0 mM final concentration) was added just before the CTC. The cell suspensions were incubated for 30 min at 37°C during which time the CTC was associated with the cell membrane. Two milliliters of CTC-labeled cell suspension were placed in cuvette and the baseline fluorescence determined. Generally, instrument settings were such that unstimulated samples had a baseline fluorescence of 85–95 arbitrary units. Excitation was at 390 nm and emission at 530 nm. Slit widths were between 6 and 8 nm. Additions of stimulant were made and the fluorescence was monitored via a recorder for 2–30 min poststimulation. Results were expressed as percent decrease in fluorescence at 1 min after addition of stimulant.

Electrophoretic Mobility

PMN (10⁷/ml KRP) were preincubated at 37°C. The stimulant was added and the cells were incubated for 10 min at 37°C in a shaking water bath. At the end of the incubation period, the cell suspensions were centrifuged at 400 g for 5 min. The cell pellets were washed twice in sorbital buffer (4 parts 5% sorbital, 1 part 1/15 M Sorensen's buffer, pH 7.2), and resuspended in sorbital buffer for final measurement of electrophoretic mobility.

Electrophoretic mobility measurements were made in a modified Northrop-Kunitz cell mounted in the vertical plane. Observation of cell movement was made at 400× with a monocular microscope mounted horizontally. Measurements were made at room temperature (approximately 23°C).

Velocities expressed are the results of the measurement of at least 10 cells/sample. The velocity of each cell after applying an electrical field was determined by measuring the time required to travel a 50-µ-distance. The polarity of the field was then reversed and the time measured in the opposite direction. The mean time to travel 50 µ was determined from the formula:

\[ t = \frac{2a(b - a)}{b} \]

where \( t \) is the actual migration time, \( a \) the migration time in one direction, and \( b \) the total migration time in both directions.

The electrophoretic mobility based on the above corrected time is expressed as µm/sec/volt-cm of electrical field. This is done to correct the velocity for any possible drift in the fluid buffer. All measurements were made at the first stationary level.

Release of Enzymes

A quantity of 1–1.5×10⁷ PMN were incubated in 0.9 ml KRP for 5 min at 37°C. Stimulating agent (100 µl) was added and incubation continued 2.5, 5, or 10 min longer. At the end of the incubation, the contents of the tube were centrifuged (400 g, 5 min, 4°C) and the cell-free supernatant was removed and held at 0°C.
until assay, which was usually performed within 2 hr. Total enzyme activities were determined on sonicates of l-ml samples of KRPG containing 10⁶ cells. Beta glucuronidase was determined by the cleavage of p-nitrophenyl-B-D-glucuronide in acetate buffer at pH 4.6 and results were expressed as nanomoles substrate converted/min/10⁶ cells. Lysozyme was performed by use of a kit from Worthington Biochemical Corp. (Freehold, N.J.), and results were expressed as microgram egg white lysozyme equivalents/10⁷ cells. Myeloperoxidase (MPO) was assayed by the method of Paul et al. and results were expressed as nanomoles substrate converted/min/10⁶ cells. Lactic acid dehydrogenase (LDH) was determined by the method of Bergmeyer and Bernt and expressed as nanomoles NAD reduced/min/10⁷ cells. Activities are indicated in the footnotes of Table 5. Results of replicate samples were averaged and expressed as percent total enzyme released.

Aggregation

Aggregometry was performed as a modification of methods described by Craddock et al. A standard platelet aggregometer (Payton, Model 300B) with dual pen recorder was used. To a siliconized cuvette containing a Teflon stirbar revolving at 600 rpm, 0.45 ml of a suspension containing 10⁶ PMN/ml KRP was added. After a 2-min delay to allow warming of cells to 37°C, 10 µl aggregant was added and the resulting changes in light transmission recorded as ΔT. To provide the necessary amplification for a well defined aggregation wave, the aggregometer recorder system was calibrated with fresh PMN suspension diluted to a 33% volume with KRP. Quantitation of PMN aggregation during the initial 3 min was made using a Lietz compensating optical polar planimeter (Model 3651-30) and results expressed in square centimeters. The variability and total aggregation by PMN during the 4-min interval did not exceed 15% on the same day or on subsequent days.

PMN Adherence

Cultures of human endothelial cells were prepared from human umbilical veins according to the modification by Czervionke et al. of the method of Jaffe et al. Cells were suspended in medium 199, with Earle's salts (M-199): 9.87 g/liter medium 199 powder, 2 mM L-glutamine, 0.05 g/liter neomycin sulfate, 0.0025 g/liter fungizone (amphotericin-B), and 2.2 g/liter NaHCO₃, pH 7.4 (M-199 with 20% human serum in a ratio of 8 parts M-199:2 parts human serum). The cells were seeded in 35 x 10 mm Petri dishes (Falcon Lab., Division of Becton, Dickinson & Co., Oxford, Calif.) and incubated in a 5% CO₂ atmosphere at 37°C. After 24 hr, the monolayers were washed with fresh M-199 with 20% human serum and then layered with 2.5 ml of the same medium. Confluent primary endothelial monolayers, containing 0.7-0.8 x 10⁶ cells, were used for adherence studies 4-7 days after seeding. Cell counts were determined with a hemocytometer after suspension of the cells with 0.25% trypsin-0.05% trisodium EDTA in phosphate-buffered saline. PMN from Ficoll gradient were washed twice in KRP, and then suspended in autologous human serum at a final concentration of 0.8-2 x 10⁶ PMN/ml. After removal of the M-199 from the tissue cultures, the plates were washed twice with Dulbecco's phosphate-buffered saline, pH 7.4, and then 1 ml of PMN suspension was added to triplicate plates just covering the 35-mm diameter surfaces. The tissue culture–PMN overlay was incubated at 37°C for 15 min; the suspension was then aspirated. Comparison of the PMN counts before and after incubation permitted calculation of the percentage of PMN adhering to the monolayers. In experiments evaluating the effect of PAF on the tissue culture–PMN overlay, PAF was added directly to the experimental plates at concentrations indicated in Table 6. A gentle agitation followed to assure adequate mixing.

Statistics

Statistical significance was determined by t test comparing PAF-treated samples with controls incubated with equivalent aliquots of 0.25% BSA in 0.9% NaCl, the suspending medium for PAF.

RESULTS

Metabolic Responses of PMN to PAF Stimulation

A variety of stimuli elicited the respiratory burst from PMN. We evaluated the ability of PAF to exert this effect by measuring O₂ utilization and O₂ production. Oxygen utilization in cells incubated with PAF (10⁻⁹-10⁻⁶M) was not significantly different from that of nonstimulated PMN (e.g., with 10⁻⁶M PAF, 15.9 ± 2.5 nmole O₂/5 min/10⁶ cells; without stimulation, 18.1 ± 4.5, p = 0.46). Likewise, little O₂ production occurred unless relatively high concentrations were used and the cells were pretreated with cytochalasin-B (5 µg/ml) (Table 1). Thus, PAF alone showed little effect on the PMN respiratory burst. However, if PMN were treated with PAF (10⁻⁹-10⁻⁶M) for 5 min before addition of FMLP (10⁻⁷M), the cells showed a significant increase in O₂ utilization (Table 2). Therefore, although PAF alone cannot elicit the respiratory burst, it does appear to “prime” the cells in their response to the chemoattractant, FMLP.

The ability of PAF to stimulate oxygenation of arachidonic acid was also evaluated. Cells were labeled with ¹⁴C-arachidonic acid and stimulated with 10⁻⁷M PAF for 5 min. Substances that comigrated with authentic 5-HETE showed a marked increase in radioactivity compared to controls (without PAF 251 ± 51 dpm/10⁶ cells; with 10⁻⁷M PAF, 579.8 ± 38, p = 0.001). Therefore, it appears that even brief exposure to PAF promotes production of lipoxygenase endproducts.

Table 1. Effect of PAF on Release of Superoxide Ion From Human PMN

<table>
<thead>
<tr>
<th>PAF Concentration*</th>
<th>Superoxide Release†</th>
<th>+ Cytochalasin-B</th>
<th>+ Cytochalasin-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>ND†</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5 x 10⁻⁷ M</td>
<td>6.1 ± 0.3§</td>
<td>18.0 ± 2.4</td>
<td></td>
</tr>
<tr>
<td>5 x 10⁻⁸ M</td>
<td>11.5 ± 4.5</td>
<td>35.2 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

*Cells were incubated in KRPG as described in Materials and Methods. Some samples received cytochalasin-B (5 µg/ml). After 5 min incubation at 37°C, 100 µl of stimulant was added. Samples receiving no PAF had 100 µl of 0.9% NaCl with 0.25% BSA, the solvent for PAF.
†Averages and standard deviations from two experiments performed.
‡ND, not detected.
§Although the values with PAF are very low, we stimulated these cells also with zymosan opsonized with human serum (1.6 mg/ml final concentration). These zymosan-stimulated samples showed a 5-min release of 253 n mole O₂; addition of 5 x 10⁻⁷ M PAF to zymosan-stimulated samples had no significant effect on O₂ production at 5 min.
Membrane and Cell Surface Responses of PMN to PAF

Chlorotetraycine HCl (CTC) has been employed to monitor translocation of calcium from membrane locations in mammalian cells. Rabbit PMN labeled with CTC exhibit an immediate loss of fluorescence upon stimulation with chemotactic agents such as FMLP or C5a, and this has been interpreted as indicating a removal of calcium from the PMN membrane. We have employed CTC-labeled human cells in an effort to compare the effects of PAF and of FMLP on fluorescence intensity. Exposure of human PMN to 2.5 x 10^-7 M FMLP causes an immediate fluorescence loss (Fig. 1A), as does exposure to 2.5 x 10^-8 M PAF (Fig. 1B). Therefore, it appears that both compounds have similar effects on membrane-associated cations.

However, the means by which the effect is triggered is different for the two compounds. Cells stimulated with FMLP remain refractory to the chemotactic agent for several minutes following the initial exposure, but if the second stimulation is PAF, a typical drop in fluorescence is seen (Fig. 1C). The same is true for PAF-stimulated cells that cannot be restimulated with FMLP but will respond to FMLP (Fig. 1D). The magnitude of fluorescence change in CTC-labeled PMN stimulated with PAF varies with PAF concentration from 10^-10 M to 10^-8 M (Table 3).

We have employed electrophoretic mobility of PMN for studies of changes in cell surface charge. After exposure to PAF, PMN have decreased mobility (Table 4). This change in mobility is consistent with a decrease in the net negative charge on the cell surface. The magnitude of the decrease in mobility is similar to that found for cells treated with FMLP in the absence of cytochalasin-B (e.g., unstimulated controls, 2.09 ± 0.031 μm/sec/volt-cm; with 10^-7 M FMLP, 1.98 ± 0.05; with 10^-8 M PAF, 1.94 ± 0.11).

Functional Responses of Human PMN to PAF

PAF is chemotactic for PMN. We wished to determine if PAF also elicited other functional responses...

Table 2. Effect of PAF Incubation* on O2 Utilization of Human PMN Stimulated With FMLP, 10^-8 M

| PAF Concentration | nmol O2/5 min/10^9 Cells | p Value
|-------------------|--------------------------|--------
| None              | 32.3 ± 2.5               | —      |
| 10^-8 M           | 52.1 ± 2.2               | 0.0015 |
| 10^-7 M           | 65.1 ± 4.3               | 0.0012 |
| 10^-6 M           | 107.1 ± 4.5              | 0.001  |
| 10^-5 M           | 139.6 ± 16.3             | 0.001  |

*1 x 10^8 PMN in KRPG and 1 mM azide were treated 5 min at 37°C with PAF at the indicated dosages and then stimulated with FMLP (10^-7 M). Results are for one of two experiments performed. Averages and standard deviation of three replicates are shown.

Table 3. Percent Decrease in Fluorescence of CTC-Labeled PMN Stimulated With PAF*

<table>
<thead>
<tr>
<th>PAF Concentration</th>
<th>Percent Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 x 10^-11 M</td>
<td>0.6 ± 0.07</td>
</tr>
<tr>
<td>2.5 x 10^-10 M</td>
<td>2.9 ± 1.5</td>
</tr>
<tr>
<td>2.5 x 10^-9 M</td>
<td>4.2 ± 1.3</td>
</tr>
<tr>
<td>2.5 x 10^-8 M</td>
<td>5.9 ± 0.3</td>
</tr>
<tr>
<td>2.5 x 10^-7 M</td>
<td>6.1 ± 0.9</td>
</tr>
</tbody>
</table>

*Two milliliters of cells in Hepes balanced salts (3 x 10^-4/m1) plus 1.2 mM CaCl2 were incubated with 10 μM CTC for 30 min. Baseline fluorescence was determined, 5 μl PAF dilution was added, and fluorescence monitored.

†Range was selected so that baseline fluorescence (in arbitrary units) was between 85 and 95. Percent was calculated from decrease in fluorescence units 1 min after PAF stimulation. Results are mean and standard deviation of triplicate determinations.

Table 4. Effect of PAF on Electrophoretic Mobility of Human PMN

<table>
<thead>
<tr>
<th>PAF Concentration</th>
<th>μm/sec/volt-cm*</th>
<th>p Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.09 ± 0.027</td>
<td>—</td>
</tr>
<tr>
<td>10^-8 M</td>
<td>2.01 ± 0.031</td>
<td>0.04</td>
</tr>
<tr>
<td>10^-7 M</td>
<td>1.94 ± 0.076‡</td>
<td>0.04</td>
</tr>
<tr>
<td>10^-6 M</td>
<td>1.90 ± 0.043</td>
<td>0.005</td>
</tr>
</tbody>
</table>

*Each value represents mean and standard deviation of 10 or more individual determinations

†One-tailed comparison of sample means of at least 2 experiments.

‡Changes with PAF at 10^-7 M are similar to those obtained with FMLP at the same concentration, 1.98 ± 0.05 μm/sec/volt-cm.
The initial cell suspension is diluted 1:3. See Materials and Methods for details.

The aggrogometer is set so that full aggregation of human PMN in response to PAF (10^-6 M) significantly increased the number of PMN remaining associated with the monolayers. An increase of PAF to 10^-8 M enhanced this effect but higher concentration did not further promote adherence.

**DISCUSSION**

The synthetic peptide, FMLP, and the phospholipid mediator of anaphylaxis, PAF, both serve as chemoattractants for PMN. We have shown in our present study that PAF, like FMLP, also influences a variety of PMN responses. However, its effects are distinct from those of the peptide in some instances.

PAF itself does not initiate the respiratory burst in non-cytochalasin-B-treated PMN. Unlike FMLP, which elicits a brief but measurable consumption of O2 and production of O2-, PAF fails to show significant effects on PMN respiration. Interestingly, PAF treatment of PMN does promote greater O2 utilization when cells are subsequently stimulated with FMLP. In addition, PAF stimulated arachidonic acid metabolism, Thus, in the inflammatory milieu, PAF may heighten the oxidative response of PMN and promote their mobility through increased production of the chemoattractant HETE.

PAF, like FMLP, exerts influence on the PMN membrane. The results with CTC-labeled cells suggest that divalent cations are dislocated from membrane locations upon PAF stimulation. Similar studies of FMLP and C5a effects on CTC-labeled PMN led to the conclusion that calcium was displaced during the initial ligand-receptor binding, and more recently, cytochalasin-D as well as *Escherichia coli* were also found to elicit fluorescence changes. In the latter instance, the intracellular calcium antagonist, 8-(N,N-diethyl-amino)-octyl-3,4,5-trimethoxybenzoate, prevented the changes in CTC fluorescence, lending support to the idea that calcium was involved. CTC...

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**Table 5. Effect of PAF on Release of Enzymes From Human PMN**

<table>
<thead>
<tr>
<th>PAF Concentration</th>
<th>Lysozyme - cyt-B</th>
<th>+ cyt-B</th>
<th>B-glucuronidase - cyt-B</th>
<th>+ cyt-B</th>
<th>MPO - cyt-B</th>
<th>+ cyt-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.2 ± 0.1</td>
<td>7.3 ± 3.2</td>
<td>4.4 ± 0.1</td>
<td>5.4 ± 1.3</td>
<td>0.9 ± 0.1</td>
<td>1.4 ± 0.6</td>
</tr>
<tr>
<td>1 x 10^-7</td>
<td>2.2 ± 1.5</td>
<td>11.8 ± 9.2</td>
<td>4.5 ± 0.4</td>
<td>15.1 ± 11.5</td>
<td>1.3 ± 0.5</td>
<td>5.7 ± 5.2</td>
</tr>
<tr>
<td>5 x 10^-7</td>
<td>2.7 ± 0.8</td>
<td>16.6 ± 2.4</td>
<td>5.3 ± 0.4</td>
<td>11.3 ± 4.0</td>
<td>1.4 ± 0.6</td>
<td>7.6 ± 7.1</td>
</tr>
<tr>
<td>1 x 10^-6</td>
<td>4.0 ± 1.1</td>
<td>17.8 ± 2.4</td>
<td>6.8 ± 2.3</td>
<td>12.1 ± 2.8</td>
<td>1.8 ± 1.1</td>
<td>8.9 ± 6.1</td>
</tr>
<tr>
<td>5 x 10^-6</td>
<td>5.1 ± 0.3</td>
<td>16.6 ± 0.8</td>
<td>6.2 ± 0.2</td>
<td>10.3 ± 0.6</td>
<td>1.7 ± 0.6</td>
<td>5.1 ± 0.5</td>
</tr>
</tbody>
</table>

* Averages and standard deviation of results from 2 experiments performed.
+ Cells were incubated in KRPG at 10^6 cells/ml for 5 min with and without cytochalasin-B (5 μg/ml). PAF was added at the indicated concentrations, and incubation continued an additional 5 min. Enzyme assays were performed on cell-free supernatants from the suspensions.
+ Total enzyme values were for lysozyme: 25.2 ± 1.2 μg egg white lysozyme equivalent per 10^7 cells; for B-glucuronidase 3.47 ± 0.38 mmole substrate converted/min/10^7 cells; for MPO 1.76 ± 0.4 mmole substrate converted/min/10^7 cells. LDH release was less than 5% for all PAF concentrations tested with or without cytochalasin-B.

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**Fig. 2.** Aggregation of human PMN in response to PAF (10^-6 M). T is in arbitrary units. The aggregometer is set so that full scale represents the change in light transmission obtained when the initial cell suspension is diluted 1:3. See Materials and Methods for details.
fluorescence may be affected by factors other than the availability of divalent cations. A difficulty in interpretation of CTC data is the possibility that the dye might be complexed with cations and then seek a membrane location rather than serving as a direct probe of membrane sites already occupied by cations. Therefore, it is not possible to conclude with certainty that all fluorescence changes are directly related to loss of cations specifically bound to the membrane. Whatever the source of the cations involved, the effects of PAF appear to be very similar to those of FMLP. However, the PAF response appears to be mediated via a mechanism distinct from that for FMLP. Repeated stimulation by PAF or by FMLP is not possible but sequential stimulation by first one and then the other is possible.

Assuming that fluorescence loss in CTC-labeled PMN is due to calcium being released from membrane stores, we may postulate that the cations becomes available for regulatory function. Such an effect may be instrumental in activating certain cell processes. For example, Ca\(^{2+}\)-mediated activation of a phospholipase A\(_2\) could increase the levels of free arachidonic acid and thus contribute to the increased arachidonate metabolism following PAF stimulation.

Our electrophoretic mobility studies show that PAF-treated PMN do have decreased mobility, a change that is consistent with decreased net negative charge at the cell surface. Others employing a similar system have also found evidence of decrease in net negative surface charge density of PMN following treatment with C5a or FMLP. Displacement of calcium, as suggested by the CTC data discussed above, might contribute to changes in cell surface charge. Many other events occurring during PMN stimulation probably also alter the net charge. Cation flux occurs, and positively charged proteins are discharged from granules. The relative contribution of these factors, or of others as yet undescribed, to PMN surface charge change remains unknown.

A diminished negative charge at cell surface should theoretically enhance cell/cell interaction, and indeed we do demonstrate, as have others, enhanced PMN autoaggregation. In contrast to our results and those of O’Flaherty et al. and Shaw et al., O’Donnell et al. showed inhibition of FMLP-mediated aggregation when human PMN were pretreated with PAF. The latter source of PAF was methanolic extracts of antigen-challenged rabbit basophils. The human PMN also showed diminished aggregation responses to A23187 and zymosan-activated serum after treatment with this PAF. The sources, and possibly also the relative purities, of the PAF preparations for each of these studies were different, and these differences may account for the apparently conflicting effects of PAF on PMN aggregation. Different tissues within a single species may give rise to PAF-like substances that are not identical in their effects on platelet release of serotonin.

Consistent with our results of enhanced PMN autoaggregation following PAF treatment is our finding that adherence of the phagocytes to endothelial cells is increased in the presence of PAF. One of the characteristics of in vivo administration of PAF is neutropenia. This loss of PMN from the peripheral circulation could reflect their increased adherence to vessel walls. Likewise, the aggregated PMN may be sequestered in capillaries. Craddock and others have suggested that such leukoaggregation contributes to respiratory distress in disease states where C5a is present in the circulation. Similarly, in vivo effects accompanying IgE-mediated anaphylaxis may be related to PAF activation of PMN.

Although PAF exerts several effects similar to those elicited by the chemotactic peptide FMLP, it does show distinct differences. Our CTC studies show that the membrane structures interacting with PAF are probably distinct from those for FMLP. Further, PAF brings about changes in arachidonic acid metabolism and cell surface charges and promotes aggregation and adherence without triggering production of reduced oxygen by products such as superoxide ion. It is

### Table 6. PAF Effects on PMN Self-aggregation and Adherence to Endothelial Cells

<table>
<thead>
<tr>
<th>PAF Concentration</th>
<th>Aggregation (cm/4 min)*</th>
<th>p Value</th>
<th>Adherence (% Adherent Cells)†</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.2 ± 1.0</td>
<td>—</td>
<td>45.6 ± 4.1</td>
<td>—</td>
</tr>
<tr>
<td>10 (^{6}) M</td>
<td>9.1 ± 1.8</td>
<td>0.0005</td>
<td>54.2 ± 6.6</td>
<td>0.0025</td>
</tr>
<tr>
<td>10 (^{7}) M</td>
<td>26.9 ± 6.9</td>
<td>0.0005</td>
<td>71.0 ± 10.6</td>
<td>0.0005</td>
</tr>
<tr>
<td>10 (^{8}) M</td>
<td>37.3 ± 11.3</td>
<td>0.0005</td>
<td>72.0 ± 10.9</td>
<td>0.0005</td>
</tr>
<tr>
<td>10 (^{9}) M</td>
<td>61.3 ± 16.8</td>
<td>0.0005</td>
<td>71.7 ± 11.3</td>
<td>0.0005</td>
</tr>
</tbody>
</table>

*0.45 x 10\(^{6}\) cells in 0.45 ml KRP were incubated for 2 min at 37°C. PAF indicated dosage was added in 10 μl volume and the aggregation wave recorded. The area under the curve was determined by planimeter and expressed in square centimeters. Results are means and standard deviation for replicate samples.

†One milliliter PMN suspensions (0.8-2.0 x 10\(^{7}\)/ml autologous human serum) were added to Petri plates containing confluent primary endothelial cell monolayers. The plates were incubated at 37°C for 15 min. The suspending fluid was aspirated and numbers of PMN determined.
possible with this compound, therefore, to dissociate the respiratory burst from other PMN responses. Relatively low concentrations of PAF elicit PMN responses. Nanomolar quantities enhance O2 utilization, promote CTC fluorescence changes, and bring about aggregation and adherence. Thus, this compound, believed to be of central importance in IgE-mediated anaphylaxis, is capable of exerting profound effects of PMN function at concentrations likely to be available in vivo.

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