Vol. 94, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 16, 1980 Pages 881-888

NOVEL EFFECTS OF 1-O-HEXADECYL-2-ACYL-SN-GLYCERO-3-PHOSPHORYLCHOLINE MEDIATORS ON HUMAN LEUKOCYTE FUNCTION: DELINEATION OF THE SPECIFIC ROLES OF THE ACYL SUBSTITUENTS

Edward J. Goetzl, Claudia K. Derian, Alfred I. Tauber, and Frank H. Valone

From the Howard Hughes Medical Institute Laboratory at Harvard Medical School, and the Departments of Medicine, Harvard Medical School, and the Robert B. Brigham Hospital and Peter B. Brigham Hospital Divisions of the Affiliated Hospitals Center, Inc., Boston, Massachusetts 02115

Received April 29,1980

SUMMARY

Analogues of 1-O-hexadecyl-2-acyl-sn-glycero-3-phosphorylcholine with different 2-acyl substituents are chemotactic for human neutrophils and mononuclear leukocytes and influence other leukocyte functions. The double bond of the 2-maleyl-analogue results in increased chemotactic potency and a free carboxyl-group endows the 2-maleyl- and 2-succinyl-analogues with the capacity to increase neutrophil adherence, while only the 2-acetyl-analogue exhibits secretagogue activity for lysosomal enzymes. Each of the analogues profoundly alters the responsiveness of neutrophils to other chemotactic stimuli without affecting random migration. The distinct profile of functions of each analogue suggests that studies of this new class of leukotactic mediators may contribute to the elucidation of the mechanisms of leukocyte activation.

INTRODUCTION

A variety of lipid factors, including some oxygenation products of arachidonic acid (1,2) and lipoproteins extracted from bacterial sources (3), exhibit chemotactic and chemokinetic activity for leukocytes. The recent demonstration that 1-O-alkyl-2-acetyl-sn-glycero-3-phosphorylcholine possesses platelet-activating activity (4,5) suggested that 1-alkyl ether phospholipids, which are present in only trace quantities in most mammalian cells, might also stimulate leukocyte function. The current data show that the 2-acetyl-, 2-maleyl-, 2-succinyl-, and 2-phthalyl-analogues of 1-O-hexadecyl-2-acyl-sn-glycero-3-phosphorylcholine are chemotactic for human leukocytes and modulate other leukocyte functions.

MATERIALS AND METHODS

Chemotactic chambers (Adaps, Inc., Dedham, Mass.), filters with 3 μm or 8 μm pores (Sartorius, Göttingen, West Germany), Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), calf thymus DNA (Worthington

0006-291X/80/110881-08$01.00/0

Biochemical Corp., Freehold, N.J.), 3,5-diaminobenzoic acid, acetic anhydride, maleic anhydride, succinic anhydride, phthalic anhydride (Aldrich Chemical Co., Inc., Milwaukee, Wisc.), [^{3}H]acetic anhydride, [^{3}H]succinic anhydride (New England Nuclear Co., Boston, Mass.), [^{3}H]phthalic anhydride (ICN Chemical and Radioisotope Division, Irvine, Calif.), 60 mm diameter plastic Petri dishes (Falcon Plastics, Oxnard, Calif.), NADPH, ferricytochrome C (Type VI), superoxide dismutase, Micrococcus lysodeikticus, a reagent kit for assaying β-glucuronidase (Sigma Chemical Co., St. Louis, Mo.), silicic acid (Mallinckrodt Chemical Works, Inc., St. Louis, Mo.), silica gel G and silica gel H 250 μm layer plates (Analtech, Inc., Newark, Del.), and organic solvents (Burdick and Jackson Co., Muskegon, Mich.) were obtained as noted. Purified synthetic formyl-methionyl-leucyl-alanyl-phenylalanine (f-Met) was supplied by Dr. R. J. Freer (Medical College of Virginia, Richmond, Va.). Chemotactic fragments of the fifth component of human complement (C5fr) were prepared by filtration on Sephadex G-75 of human sera that had been incubated with zymosan (6).

Preparation of 1-O-hexadecyl-2-acyl-sn-glycero-3-phosphorylcholine analogues. 1-O-hexadecyl-sn-glycero-3-phosphorylcholine was prepared from the plasmalogens in extracts of fresh beef heart by silica gel G thin-layer chromatography, catalytic hydrogenation, alkaline methanolysis, and sequential chromatography on a silicic acid column and a silica gel G thin-layer plate (5). Ten μmol of 1-O-hexadexyl-sn-glycero-3-phosphorylcholine were dissolved in 1 ml of chloroform to which was added 100 μmol of acetic anhydride, maleic anhydride, succinic anhydride or phthalic anhydride, with or without 25-100 μCi of the respective radiolabeled anhydride. The mixture was heated at 60°C for 60 min under N_2 and the chloroform was extracted with 2 ml portions of methanol:water (10:9, v:v) until the aqueous phase was acid-free. The product in the chloroform layer was purified by high-pressure liquid chromatography (HPLC) utilizing a C18 reversed-phase Ultrasphere column in an Altex Model #322 system (Altex Scientific, Inc., Berkeley, Calif.) that was eluted isocratically with methanol:chloroform:water (100:10:10, v:v) (7). The extent of coupling of the acyl-groups to the ether lysophospholipid precursor was assessed by the specific radioactivity of the product; coupling efficiency ranged from 69-78% for the phthalyl-analogue to 94-99% for the acetyl-analogue. The homogeneity and purity of each derivative was demonstrated both by chromatography on silica gel G 250 μm plates that were developed with chloroform:methanol:water (65:35:6, v:v) (5) and by HPLC on a Lichrosorb Si 60 column (Hibar-II, 10 μm, EM Reagents, Cincinnati, Ohio) developed with a 20 min linear gradient from n-hexane:2-propanol:water (6:8:0.7, v:v) to n-hexane:2-propanol:water (6:8:1.4, v:v) and then isocratic elution for 20 min with the latter solvent (8). The single peak of each product was detected by assessing radioactivity, $OD_{206\ nm}$, and phosphorus content (9) in portions of each fraction.

Assessment of human leukocyte function. Human leukocytes from normal subjects were separated by centrifugation on Ficoll-Hypaque into neutrophils of over 96% purity and mononuclear leukocytes containing 16-21% monocytes and 74-81% lymphocytes (1,2,6). Leukocyte chemotaxis and chemokinesis were assayed by a modification (2,6) of the Boyden chamber micropore filter technique utilizing 3 μm pore filters for neutrophils and 8 μm pore filters for mononuclear leukocytes. Chemotaxis was evoked by a stimulus in the lower compartment alone and chemokinesis was elicited by an equal concentration of a stimulus in the lower compartment and in the leukocyte well. The chemotactic and chemokinetic responses were expressed as net leukocytes per high power field (hpf) after subtraction of the background level of migration in the absence of a stimulus. Adherence was assessed by incubating 5 x 10^6 neutrophils in 2 ml of Hanks' solution (HBSS)-0.4% ovalbumin (OA) without or with an agonist for 15 min at 37°C in a plastic Petri dish, washing

the dish twice with HBSS, and dissolving the adherent neutrophils in 1 ml of 3 g sodium lauryl sulfate/100 ml distilled water (10). The quantity of DNA in lysates of the adherent neutrophils and of 5 x 10^6 neutrophils was assayed by the diaminobenzoic acid method and adherence was expressed as a percentage of the total neutrophils added to each dish (10). Superoxide generation at 37°C was quantitated as described by measuring the superoxide dismutase-inhibitable reduction of cytochrome C (11) and oxygen consumption was determined using a chamber with an oxygen electrode (Model 53, Yellow Springs Instrument Co., Yellow Springs, Ohio) that was calibrated according to the phenylhydrazine oxidation method (12). 2.5±0.5 x 10^7 neutrophils were incubated in 1 ml HBSS-0.1% OA with 5 μg/ml of cytochalasin B for 15 min at 37°C without or with an agonist, and the tubes centrifuged at 400 g for 5 min at 4°C to sediment the leukocytes. Lysozyme and β-glucuronidase activities were assayed (10,11) in portions of the supernates and neutrophil pellet sonicates and the results were expressed as percentage enzyme release.

RESULTS

Maximal neutrophil chemotactic responses of similar magnitude were elicited by the 2-acetyl-, 2-succinyl-, and 2-maleyl-analogues of 1-O-hexadecyl-2-acyl-sn-glycero-3-phosphorylcholine at concentrations of 10^{-5} M - 10^{-4} M (Fig. 1), while the maleyl-analogue was the most potent of the factors. In contrast, the phthalyl-analogue elicited only slight neutrophil chemotaxis that was maximal at 10^{-7} M - 10^{-6} M. The maleyl-analogue also was significantly more potent than the acetyl-analogue in evoking mononuclear leukocyte chemotaxis, but the succinyl-analogue evoked only a marginal response. In two experiments with purified eosinophils from hypereosinophilic donors (13), the pattern of chemotactic stimulation was similar to that for neutrophils. The weak chemokinetic response of neutrophils to 10^{-6} M maleyl-analogue was the only one detectable with either type of leukocyte (Fig. 1), and checkerboard assays confirmed that the enhancement of leukocyte migration by the phospholipid factors was chemotactic in nature. When neutrophils were preincubated with the analogues for 15 min at 37°C and washed twice in buffer, the chemotactic responses to C5fr, 10^{-5} M acetyl-analogue and 10^{-6} M maleyl-analogue were suppressed significantly by the acetyl-analogue and enhanced significantly by the succinyl-analogue in a dose-related manner, without affecting random migration. In contrast, the maleyl- and phthalyl-analogues significantly suppressed the chemotactic responses of the neutrophils

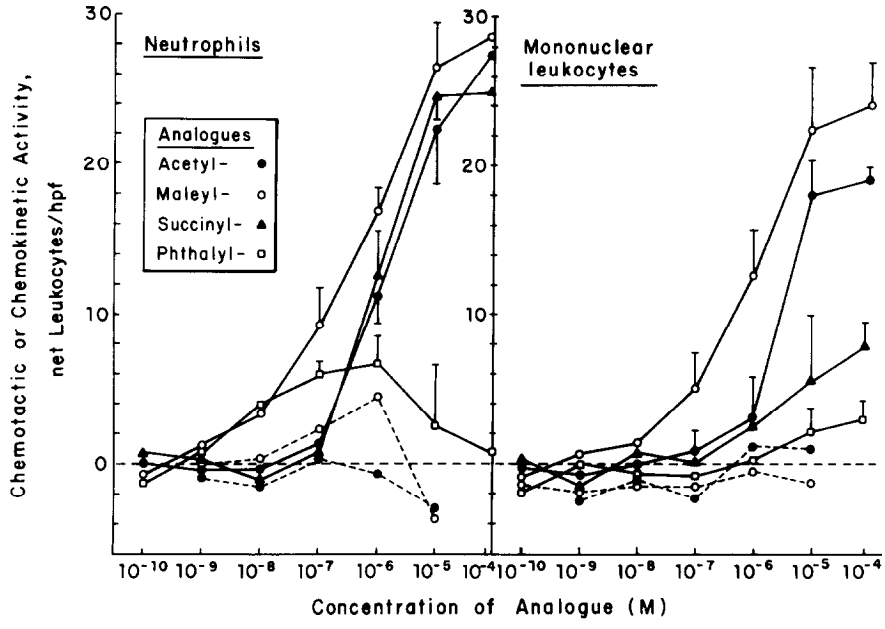

Figure 1. Human leukocyte chemotactic (—) and chemokinetic (---) activities of 1-O-hexadecyl-2-acyl-sn-glycero-3-phosphorylcholine analogues. Each point and bracket are the mean ± SD of the values from three experiments. The levels of background migration in the absence of a stimulus were 3.3, 4.1 and 2.7 neutrophils/hpf and 3.0, 4.1 and 2.5 mononuclear leukocytes/hpf. The leukocyte chemotactic response to the maleyl-analogue was significantly greater than that to the acetyl-analogue at concentrations of 10^{-7} M ($p < 0.01$) and 10^{-6} M ($p < 0.05$) for neutrophils, and 10^{-6} M ($p < 0.01$) for mononuclear leukocytes.

at 10^{-6} M and enhanced the responses at 10^{-10} M, irrespective of the specific chemotactic stimulus (Fig. 2).

The adherence of neutrophils to plastic Petri dishes was enhanced significantly only by 10^{-5} M succinyl-analogue and 10^{-5} M and 10^{-6} M maleyl-analogue (Table I). The release of β-glucuronidase and lysozyme from neutrophils was increased significantly only by the highest concentrations of the acetyl-analogue, while 10^{-5} M maleyl-analogue stimulated the release of β-glucuronidase, but not lysozyme (Table I). The generation of superoxide and the consumption of oxygen by the neutrophils were not altered significantly by any of the analogues, as contrasted with the more than 3-fold rise and the more than 50-fold rise in superoxide generation elicited by 10^{-5} M f-Met and by PMA, respectively. In two experiments, 10^{-5} M and 10^{-6} M concentrations of the analogues were not cytotoxic for neutrophils after 30 min at 37°C,

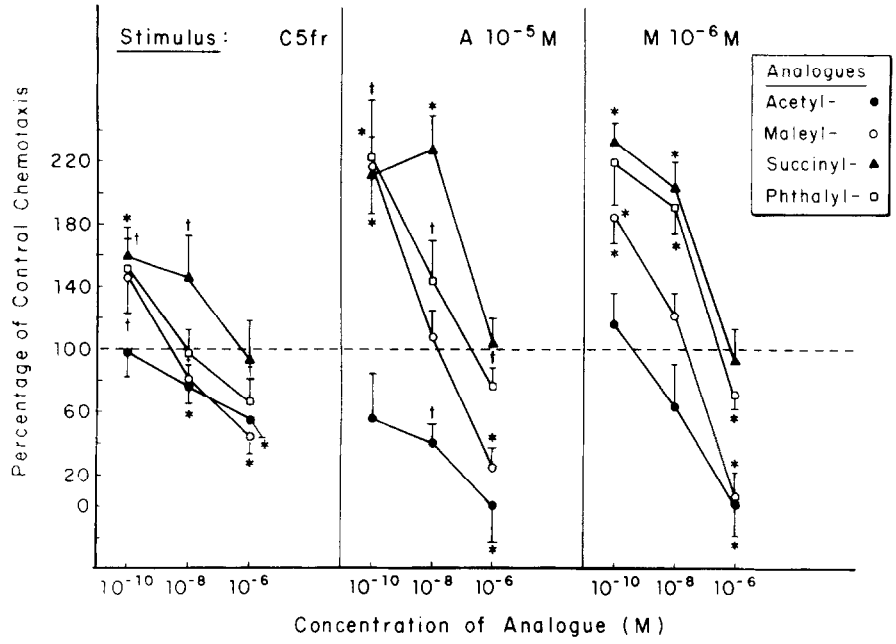

Figure 2. Modulation of human neutrophil chemotaxis by 1-O-hexadecyl-2-acyl-sn-glycero-3-phosphorylcholine analogues. Each point and bracket are the mean ± SD of the values from three experiments. The chemotactic responses of control neutrophils preincubated in buffer alone (100%) were 36.8, 47.1, and 48.0 net neutrophils/hpf, respectively, for C5fr, 20.4, 21.8, and 26.7 for 10^{-5} M acetyl-analogue and 26.5, 32.8, and 27.6 for 10^{-6} M maleyl-analogue. The level of significance of the enhancement (>100%) or suppression (< 100%) of the chemotactic responses is denoted by symbols: * = $p < 0.01$ and + = $p < 0.05$.

as assessed by trypan blue dye exclusion which revealed mean levels of viability of 93% or greater in each instance.

DISCUSSION

Each of the 2-acyl-analogues of 1-O-hexadecyl-2-acyl-sn-glycero-3-phosphorylcholine exhibited a different profile of human leukocyte-directed activities, indicating that the 2-acyl-substituent is a functionally critical determinant. The double bond of the maleyl-analogue resulted in increased chemotactic potency (Fig. 1) and a free carboxyl-group endowed the maleyl- and succinyl-analogues with the capacity to increase neutrophil adherence (Table I), while only the acetyl-analogue possessed lysosomal enzyme secretagogue activity (Table I). At concentrations below the chemotactic range,

TABLE I

The Effects of 1-O-Hexadecyl-2-Acyl-sn-Glycero-3-Phosphoryl-Choline Analogues on Human Neutrophil Functions other than Migration

	Concentration of Analogue (M)										
		Acetyl-					Maleyl-				
	Buffer	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Percentage of adherence*	38.0± 7.8	44.0± 9.3	41.7± 9.2	40.4± 9.5	--	39.8± 6.8	52.8± 3.9^{+}	44.6± 9.6^{+}	40.1± 5.2	--	37.4± 5.4
Percentage of release of ß-glucuronidase*	1.4± 0.1	4.5± 0.2$^{\#}$	2.1± 0.1$^{\#}$	1.7± 0.2	1.5± 0.3	--	2.6± 0.7^{+}	1.9± 0.5	1.7± 0.4	1.6± 0.2	--
Percentage of release of lysozyme*	10.3± 2.5	17.1± 2.4^{+}	14.3± 1.1	10.4± 2.2	10.2± 2.4	--	12.3± 2.1	11.5± 1.9	9.8± 3.0	9.9± 2.6	--

	Concentration of Analogue (M)											
		Succinyl-					Phthalyl-					
	Buffer	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	f-Met,‡ 10^{-5} M
Percentage of adherence*	38.0± 7.8	59.4± 9.8$^{\#}$	38.0± 7.3	38.7± 5.2	--	36.6± 5.4	37.4± 5.4	38.6± 1.8	37.2± 4.7	--	38.3± 2.9	53.7±11.2
Percentage of release of ß-glucuronidase*	1.4± 0.1	2.7± 1.0	1.7± 0.2	1.6± 0.1	1.6± 0.3	--	3.4± 1.3	1.6± 0.3	1.6± 0.4	1.5± 0.4	--	21.4±2.9$^{\#}$
Percentage of release of lysozyme*	10.3± 2.5	11.6± 3.1	9.5± 2.0	10.0± 2.5	10.1± 2.3	--	14.1± 3.6	10.7± 2.4	10.2± 2.7	10.1± 2.6	--	46.6±6.4$^{\#}$

* - Each value is the mean±SD of the results of three experiments with neutrophils from different donors. Statistical analyses employed the two sample t test for enzyme release and the paired t test for adherence, as the control values for the latter assay exhibited the widest range. The symbols used to designate levels of statistical significance are: + = $p < 0.05$ and # = $p < 0.01$.

‡ - formyl-methionyl-leucyl-alanyl-phenylalanine

three of four factors in the present series significantly enhanced the chemotactic response of neutrophils to other stimuli (Fig. 2), without altering random migration.

Recent studies have suggested that 1-O-hexadecyl/octadecyl-2-acetyl-sn-glycero-3-phosphorylcholine may be the principal constituent of the platelet activating factor (PAF) released by IgE-mediated stimulation of rabbit basophils in vitro and in vivo (4,5). The purified PAF and the synthetic phospholipid exhibited identical mobility on thin-layer plates, evoked rabbit platelet aggregation and release of serotonin of comparable magnitude at concentrations of 10^{-11} M - 10^{-9} M, were susceptible to inactivation by alkaline methanolysis and were restored to full activity by reacylation with acetic anhydride (4,5). Aggregation of washed human platelets by the acetyl-analogue of 1-O-hexadecyl-2-acyl-sn-glycero-3-phosphorylcholine, in the absence of the 1-O-octadecyl-compound, is not detectable at concentrations below 3×10^{-8} M and is maximal at 3×10^{-7} M - 10^{-6} M. Thus with human cells the potency of the 1-alkyl ether phospholipids as leukotactic factors is similar to their potency as platelet-activating factors. This series of phospholipids represents a novel class of principles which activate several types of human cells, but cannot as yet be assigned a functional specificity as natural mediators of anaphylactic reactions in humans.

ACKNOWLEDGEMENTS

This work was partly supported by grants #HL-19777 and #AI-15116 from the National Institutes of Health.

REFERENCES

1. Goetzl, E. J., and Gorman, R. R. (1978) J. Immunol. 120, 526-531.
2. Goetzl, E. J., Brash, A. R., Tauber, A. I., Oates, J. A., and Hubbard, W. C. (1980) Immunology 39, 491-501.
3. Tainer, J. A., Turner, S. R., and Lynn, W. S. (1975) Am. J. Pathol. 81, 401-410.
4. Pinckard, R. N., Farr, R. S., and Hanahan, D. J. (1979) J. Immunol. 123, 1847-1857.

5. Demopoulos, C. A., Pinckard, R. N., and Hanahan, D. J. (1979) J. Biol. Chem. 253, 9355-9358.
6. Goetzl, E. J., and Hoe, K. Y. (1979) Immunology 37, 407-418.
7. Porter, N. A., Wolf, R. A., and Nixon, J. R. (1979) Lipids 14, 20-24.
8. Geurts Van Kessel, W. S. M., Hax, W. M. A., Demel, R. A., and DeGier, J. (1977) Biochim. Biophys. Acta 486, 524-530.
9. Bartlett, G. R. (1959) J. Biol. Chem. 234, 466-468.
10. Bockenstedt, L. K., and Goetzl, E. J. (1980) J. Clin. Invest., in press.
11. Tauber, A. I., and Goetzl, E. J. (1979) Biochemistry 18, 5576-5584.
12. Misra, H. P., and Fridovich, I. (1976) Anal. Biochem. 70, 632-638.
13. Goetzl, E. J., Weller, P. F., and Sun, F. F. (1980) J. Immunol. 124, 926-933.