

# Stimulation of Human Eosinophil and Neutrophil Polymorphonuclear Leukocyte Chemotaxis and Random Migration by 12-L-Hydroxy-5,8,10,14-Eicosatetraenoic Acid

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**ABSTRACT** A human platelet lipoxygenase-generated product of arachidonic acid, identified by thin-layer chromatographic and mass spectrometric properties as 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE), was selectively chemotactic *in vitro* for human polymorphonuclear leukocytes (PMN), as compared to mononuclear leukocytes, with a preference for eosinophils. Preincubation of PMN with partially-purified HETE at peak chemotactic concentrations of 8–24  $\mu\text{g/ml}$  reduced their random and chemotactic migration and stimulated the activity of their hexose monophosphate shunt; minimally chemotactic concentrations of 0.03–1  $\mu\text{g/ml}$  enhanced PMN random migration without influencing other functions. HETE may thus be capable of preferentially attracting eosinophils to foci of tissue reaction associated with platelet activation.

## INTRODUCTION

A cyclo-oxygenase in blood platelets, which is activated by platelet aggregation, converts arachidonic acid to a variety of endoperoxides, thromboxanes, and prostaglandins of differing stabilities and activities (1, 2). A platelet lipoxygenase, also activated by platelet aggregation and representing a major pathway of arachidonic acid metabolism, contrasts with the cyclo-oxygenase in generating, through a hydroperoxide intermediate, a single stable product termed 12-L-

hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) (3).<sup>1</sup> HETE apparently lacks many of the physiologic and regulatory activities characteristic of the prostanoate derivatives (1–4). A product of arachidonic acid, prepared both by photo-oxidation and treatment with lipoxygenase, has shown chemotactic activity *in vitro* for human polymorphonuclear leukocytes (PMN) (5, 6), whereas no consistent leukotactic activity has been observed for the products of the cyclooxygenase system (5–7). This leukotactic product co-chromatographed with a standard of highly purified HETE on thin-layer plates and was specifically identified as HETE by gas-liquid chromatography and mass spectrometry (6). The partially purified HETE required a concentration gradient for its chemotactic activity and inhibited the random migration of PMN when added only to the leukocyte compartment of the chemotactic chamber (5). The current studies of HETE demonstrate its spectrum of PMN-directed activities, which are preferentially expressed for eosinophils, and document the unique ability of minimally chemotactic concentrations of HETE to enhance the random migration of human PMN.

## METHODS

Disposable polystyrene chemotactic chambers (Adaps, Inc., Dedham, Mass.) were assembled with micropore filters (Millipore Corp., Bedford, Mass.) as previously described (8). Hanks' solution and Medium 199 (Microbiological Associates,

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<sup>1</sup>Abbreviations used in this paper: HBSS-ovalbumin, Hanks' balanced salt solution made 0.4% in ovalbumin; HETE, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid; HMPS, hexose monophosphate shunt; hpf, high power field; PMN, polymorphonuclear leukocyte(s).

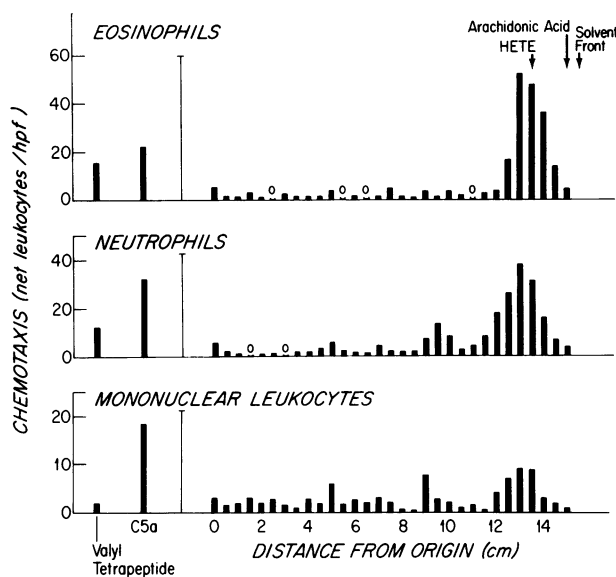


FIGURE 1 Leukocyte chemotactic activity of thin-layer chromatography fractions of platelet extract-treated arachidonic acid. The target leukocyte suspensions contained: 68% eosinophils, with 11% neutrophils and 21% mixed mononuclear leukocytes; 97% neutrophils, with 3% mixed mononuclear leukocytes; 24% monocytes, with 69% lymphocytes and 7% neutrophils. 40  $\mu$ l of each 1-ml fraction was employed as a stimulus. The concentration of valyl-tetrapeptide was 0.10  $\mu$ M and that of C5a was 5  $\mu$ g of C5 equivalent per ml.

Bethesda, Md.), ovalbumin recrystallized five times (Miles Laboratories, Inc., Elkhart, Ind.), dextran and Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), sodium diatrizoate (Hypaque, Winthrop Laboratories, New York), twice-recrystallized trypsin previously treated with diphenyl carbamyl chloride (Sigma Chemical Co., St. Louis, Mo.), [ $^{14}$ C]arachidonic acid, [1- $^{14}$ C]glucose and [6- $^{14}$ C]glucose (Amersham Searle Corp., Arlington Heights, Ill.), L-ascorbic acid and sodium lauryl sulfate (Fisher Scientific Co., Pittsburgh, Pa.), plastic 35  $\times$  100-mm Petri dishes (Falcon Plastics, Div. of Bio Quest, Oxnard, Calif.), silica gel H thin-layer chromatography plates (Analtech, Inc., Newark, Del.), and arachidonic acid (Supelco, Inc., Bellefonte, Pa.) were obtained as noted. All solvents were either Eastman Kodak Co. (Rochester, N. Y.) reagent grade or Fisher-certified (Fisher Scientific Co.) and were redistilled before use. C5a was prepared by tryptic digestion (9) of purified C5 prepared from human plasma (10).

The source of highly purified HETE was 20 mg of arachidonic acid (Nu-Chek Co., Elysian, Minn.) mixed and agitated for 1.5 h at 30°C with  $5 \times 10^{11}$  washed human platelets suspended in 672 ml of 0.1 M Tris-HCl/0.1 M  $K_2HPO_4$  buffer (pH 8.2) containing 0.10 mM indomethacin (Merck & Co., Inc., Rahway, N. J.) (3). The mixtures were then adjusted to pH 4.0 with 2 M citric acid and extracted three times with ethyl ether:n-hexane (3:1, vol/vol). The pooled extracts were washed with an equal volume of water and dried in vacuo over  $MgSO_4$ . HETE was resolved from other products in the extract by chromatography on a column of CC-4 silicic acid (Mallinckrodt Inc., St. Louis, Mo.); the purity of the HETE fraction was confirmed by thin-layer chromatography

and gas-liquid chromatography-mass spectrometry.<sup>2</sup> A double trimethylsilyl derivative of HETE-free acid was prepared by reacting Bis-(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co., Rockford, Illinois) with 1% trimethylchlorosilane for 30 min at 25°C. The excess reagents were removed with a nitrogen stream and the residue was dissolved in ethyl acetate for gas-liquid chromatography-mass spectrometry. The samples were analyzed on a Varian Associates (Palo Alto, Calif.) CH-7 system containing a 3.5-foot, VCW-98 column at 235°C. The ionization potential was 22.5 eV. Prominent peaks were observed at m/e 263, 353, and 449, corresponding to the known double trimethylsilyl fragmentation pattern of HETE.<sup>2</sup>

**Preparation of partially purified HETE.** Arachidonic acid was oxidized by both photochemical and enzymatic means (1, 5, 6). In the former procedure, 1 mg of arachidonic acid was spotted in 50- $\mu$ g portions near the lower edge of a silica gel H plate, and exposed to short wave (average 2,537 Å) ultraviolet radiation (George W. Gates & Co., Inc., Franklin Square, N. Y.) for 60 min. The plate was chromatographed in ascending fashion employing chloroform:methanol:glacial acetic acid:water (65:35:1:1, vol/vol), and eluted in 0.5-cm strips by sequential extraction with 1 ml of chloroform:methanol (2:1, vol/vol) and 1 ml of ethyl ether. The pooled eluates were evaporated at 45°C under  $N_2$  (SC/48 Sample Concentrator, Brinkman Instruments, Inc., Westbury, N. Y.) and stored under  $N_2$  at -70°C.

In the latter procedure, 0.5 mg arachidonic acid was incubated with a sonicated extract from  $2 \times 10^{10}$  purified human platelets (11) in 3 ml of 0.1 M Tris-HCl/0.05 M  $KH_2PO_4$  buffer, pH 8.0, for 120 min at 37°C (6). The HETE-containing mixture was extracted three times with 5 ml of chloroform:methanol (2:1, vol/vol) and the pooled material was evaporated, chromatographed, and processed as for the photochemically oxidized product.

**Leukocyte migration and hexose monophosphate shunt (HMPS) activity.** Blood from normal donors or patients with peripheral blood hyper eosinophilia of 28–82% was incubated for 30 min at 37°C with citrate anticoagulant and dextran to sediment the erythrocytes (8). The leukocyte-rich supernatant plasma was aspirated into tubes and centrifuged at 100 g for 10 min at room temperature. The mixed leukocyte pellet from hyper eosinophilic patients was washed and, without further purification, was resuspended in Hanks' balanced salt solution made 0.4% in ovalbumin (HBSS-ovalbumin) and 0.005 M in Tris-HCl, pH 7.4. The mixed leukocytes from normal donors were separated into neutrophils and mononuclear leukocytes on Ficoll-Hypaque (12), and each purified fraction was washed and resuspended in HBSS-ovalbumin. Leukocyte concentrations were standardized in the ranges:  $2.2 \pm 0.3 \times 10^6$  eosinophils per ml,  $2.0 \pm 0.5 \times 10^6$  neutrophils per ml, and  $3.0 \pm 0.5 \times 10^6$  mononuclear leukocytes per ml. The modified Boyden assay (13) employed micropore filters of 3- $\mu$ m pore size for eosinophils and neutrophils and of 8- $\mu$ m pore size for mononuclear leukocytes (14, 15); incubation conditions and processing of filters were as described (14, 15). Filters from two chambers known to lack a stimulus were counted initially to determine a depth near the cell front at which a background count of 2–8 leukocytes per high power field (hpf) was achieved; 10 hpf from each of the other control and experimental filters in the experiment were counted at that depth

<sup>2</sup> McGuire, J. C., R. C. Kelly, O. U. Miller, R. R. Gorman, and F. F. Sun. Biosynthesis, isolation, and characterization of 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) from human platelets. Submitted for publication.

TABLE I  
Human PMN-Directed Activities of Partially Purified HETE\*

HETE	Chemotaxis <sup>†</sup>	Random migration <sup>**</sup>	Deactivation <sup>‡‡</sup>	HMPS activity <sup>§§</sup>
$\mu\text{g equivalent/ml}$	net leukocytes/hpf	% of control	% of control chemotaxis to C5a	% of control
24	45.6±20.7	36.2±18.3†	7.2±13.3§	159.3±10.9§
8	41.8±22.5	63.3±12.7†	11.8±17.3§	133.0±4.9§
4	30.5±15.7	80.6±9.9	21.8±28.2†	109.3±9.2
2	23.7±12.4	104.5±2.5	33.5±24.5†	101.8±12.1
1	14.2±11.0	129.0±17.9†	69.6±19.4	101.7±11.7
0.3	9.3±6.6	152.1±10.4§	74.4±20.3	104.0±10.2
0.03	3.9±5.9	167.5±12.8†	75.1±21.4	102.3±13.3
0.01	3.6±3.1	111.5±24.4	103.7±13.1	90.8±4.5
0.003	1.9±2.4	101.3±17.7	99.5±6.1	100.3±5.2
Ascorbate, 2.5 mM	—	207.4±32.6§	—	202.5±34.1§

\* Values given are the mean of three experiments±1 SD. Levels of significance calculated from the paired Student's *t* test are designated: †, *P* < 0.05; §, *P* < 0.01.

† The concentration of HETE was calculated from the OD at 238 nm utilizing a standard of highly purified HETE.

‡ Mean background migration in the absence of a stimulus was 8.3 leukocytes/hpf for a standard chemotactic interval of 2.5 h.

\*\* Mean control random migration (100%) was 14.1 leukocytes/hpf after a standard migration interval of 3.5 h.

‡‡ The residual response of deactivated leukocytes is expressed as a percent of the mean control response to the C5a stimulus, at a concentration of 2.5  $\mu\text{g}$  of C5 equivalent/ml, which was 26.4 net leukocytes/hpf (100%).

§§ Mean control HMPS activity (100%) was 845 cpm/0.2 absorbancy unit 280.

without knowledge of the protocol. The chemotactic responses were expressed as net leukocytes/hpf after correction for background counts, while random migration in the absence of a chemotactic stimulus was expressed as leukocytes per hpf. Deactivation was carried out by preincubating leukocytes with chemotactic factors for 30 min at 37°C and then washing the cells three times before measurement of residual chemotactic responsiveness. Factors added to leukocytes to influence their random migration were introduced immediately before loading the cells into chemotactic chambers. The migration of leukocytes pretreated with cell additives was denoted as a percentage of the response of control leukocytes treated with buffer alone which was set at 100%.

HMPS activity of purified PMN adherent to Petri dishes was determined by measuring the rate of conversion of [1-<sup>14</sup>C]-glucose to <sup>14</sup>CO<sub>2</sub> in 1 h under conditions where no [6-<sup>14</sup>C]-glucose was converted to <sup>14</sup>CO<sub>2</sub> (16). The mean counts per minute of <sup>14</sup>CO<sub>2</sub> generated in duplicate dishes were divided by the mean OD at 280 nm of the 3% sodium lauryl sulphate solutions of the PMN in the Petri dishes to arrive at the counts per minute per 0.2 absorbancy unit 280. The effect of HETE on the HMPS activity of adherent PMN layers was expressed as a percentage of the activity observed with cells incubated in buffer alone.

## RESULTS

The oxidation products of arachidonic acid from thin-layer chromatograms were assayed for their leukocyte chemotactic activity in parallel with defined stimuli (Fig. 1). A peak of chemotactic activity for all three

cell types was detected at 12.5–14.0 cm from the origin, exhibiting a mobility comparable to that of the standard of highly purified HETE that was quantitated by its absorption at 238 nm (17). Partially purified HETE and the HETE standard had superimposable absorption spectra in the range of 225–260 nm. Dilutions of partially purified HETE were preferentially chemotactic for eosinophils with substantial activity for neutrophils and far less for mononuclear leukocytes (Fig. 1), and thus exhibited a profile of activity comparable to that of the synthetic eosinophil chemotactic factor of anaphylaxis tetrapeptide, Val-Gly-Ser-Glu (14). The dose of HETE from the peak fractions, which was equivalent to 12.5  $\mu\text{g}$  of the standard HETE by spectrophotometric analysis, gave an eosinophil response greater than that stimulated by optimal concentrations of C5a or valyl-tetrapeptide. HETE and C5a elicited an equivalent neutrophil response, but only C5a stimulated mononuclear leukocyte chemotaxis to a level near that of the other leukocyte types. The chemotactic activity of the highly purified standard HETE was examined employing normal neutrophils of 98% purity and eosinophils of 82% purity obtained from a patient with an apparent lymphoma. At HETE concentrations of 24, 8, 4, 2 and 1  $\mu\text{g/ml}$ , the eosinophil response was 43, 35, 23, 12, and 5 eosinophils/hpf over a back-

ground level of 6 eosinophils/hpf, and the neutrophil response was 24, 19, 14, 9, and 6 neutrophils/hpf over a background level of 5 neutrophils/hpf.

As neutrophils from normal donors could be readily isolated free of other cell types, the chemotactic activity of partially purified HETE for neutrophil PMN from three donors was analyzed over a concentration range of 0.003–24  $\mu\text{g}$  HETE equivalent per ml in parallel with other leukocyte-directed activities (Table I). The chemotactic activity was comparable to that of the standard HETE achieving a detectable response at 1  $\mu\text{g}/\text{ml}$  and reaching a plateau of approximately five times the background level at 8–24  $\mu\text{g}/\text{ml}$ . At concentrations of 0.03–1  $\mu\text{g}/\text{ml}$ , HETE added to the cell compartment significantly enhanced PMN random migration; the maximum level of enhancement was two-thirds of that for 2.5 mM ascorbate, an agent known to stimulate both migration and HMPS activity of PMN (16). In contrast, preincubation of PMN with partially purified HETE at peak chemotactic concentrations, followed by washing the cells, significantly reduced their subsequent response to C5a by way of a process functionally defined as chemotactic deactivation (16, 18, 19; Table I). Stimulation of the HMPS of adherent PMN, another property of some active chemotactic factors (16), was evident with HETE and, as for deactivation, was significant only at optimal chemotactic concentrations. Maximum stimulation of PMN HMPS activity was approximately 60% of the rise seen with 2.5 mM ascorbate.

## DISCUSSION

An oxidation product of arachidonic acid, prepared both by ultraviolet photolysis and by the action of a lipoxygenase derived from human platelet extracts, had potent chemotactic activity for human PMN (Fig. 1). This oxidation product was identified as HETE both by its co-chromatography on thin-layer plates with a HETE standard previously characterized by gas-liquid chromatography-mass spectrometry, and by the superimposable absorption spectra of the standard and partially purified HETE (17).<sup>2</sup> The leukocyte specificity of HETE was analyzed in parallel with two peptide chemotactic factors of known eosinophil specificity; C5a, which was derived by tryptic cleavage of highly purified human C5, and synthetic Val-Gly-Ser-Glu, one of the tetrapeptides comprising the eosinophil chemotactic factor of anaphylaxis (14). Approximately 12.5  $\mu\text{g}/\text{ml}$  of HETE elicited a greater eosinophil chemotactic response than either of the peptide factors at their respective optimal doses (Fig. 1). Both partially purified HETE and the highly purified standard HETE preparation were preferentially chemotactic for eosinophils as compared to neutrophils with only marginal activity for mononuclear leuko-

cytes, and thus exhibited a rank order of leukocyte selectivity comparable to that of the eosinophil chemotactic factor of anaphylaxis tetrapeptide (Fig. 1). Earlier reports have suggested that purified lipid chemotactic factors derived from *Escherichia coli* growth media lacked leukocyte specificity since they were equally chemotactic for human PMN and rabbit alveolar macrophages. The macrophage chemotactic specificity of crude lipid-protein complexes in the growth media before purification was attributed to the polypeptide components which were themselves chemotactically inactive (20). In contrast to this supposition, HETE exhibited a high degree of intrinsic selectivity for PMN with a preference for the eosinophil series.

Partially purified HETE possessed other leukocyte-directed activities characteristic of nonlipid chemotactic stimuli (16, 18, 19). Exposure of PMN to HETE at concentrations of 8  $\mu\text{g}/\text{ml}$  or higher, in the absence of a concentration gradient, both stimulated the activity of their HMPS and reduced their spontaneous random migration and chemotactic responsiveness (Table I). In contrast to peptide and protein chemotactic factors, which chemotactically deactivate human PMN and stimulate their HMPS at concentrations 1/5–1/20 a minimal chemotactic dose (16, 21), HETE required peak chemotactic concentrations to significantly alter these leukocyte functions. Thus, the ratio of the minimal leukotactic concentration of a chemotactic factor and the concentration required to stimulate another leukocyte function may be a specific characteristic of that factor.

A unique capability of HETE was its stimulation of PMN random migration at a concentration less than 1/200 the peak chemotactic dose. Although the basis of this effect awaits further studies, the phenomenon has been confirmed with highly purified HETE. While prior exposure of PMN leukocytes to chemotactic concentrations of HETE suppress their chemotactic responsiveness, low concentrations of HETE may enhance their responsiveness to heterologous chemotactic stimuli. The lipoxygenase pathway of human platelets, through the activity of HETE, may thus influence the chemotaxis and random migration of PMN by several mechanisms which would not be suppressed by inhibitors of cyclo-oxygenase activity.

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