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## **Brief Definitive Report**

### GENERATION OF UNIQUE MONO-HYDROXY- EICOSATETRAENOIC ACIDS FROM ARACHIDONIC ACID BY HUMAN NEUTROPHILS

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Lipoxygenases in a variety of mammalian cells transform arachidonic acid to stable mono-hydroxy-eicosatetraenoic acid (HETE) products (1-4). The lipoxygenase in mammalian platelets was the first to be described and converts arachidonic acid predominantly to 12-L-HETE (1). Other major lipoxygenase products of arachidonic acid are 5-D-HETE for rabbit polymorphonuclear (PMN) leukocytes (2), 11-HETE and 12-HETE for rat mast cells (3), and 5-HETE and 11-HETE for rabbit alveolar macrophages (4). Platelet-derived 12-L-HETE is chemotactic for human PMN leukocytes at concentrations of 0.7-25  $\mu\text{g}/\text{ml}$ , while concentrations of 0.2-2  $\mu\text{g}/\text{ml}$  exhibit chemokinetic activity and facilitate the responses of PMN leukocytes to other chemotactic factors (5, 6). To assess the possibility that endogenously produced HETE may regulate the migration of PMN leukocytes, the arachidonic acid metabolites of human neutrophil lipoxygenase have been isolated and the chemotactic and chemokinetic properties have been defined for the quantitatively predominant products.

#### Materials and Methods

Blind-end acrylic chemotactic chambers with a 0.2-ml stimulus compartment and a 0.5-ml leukocyte well (Neuro Probe, Inc., Bethesda, Md.), 3- $\mu\text{m}$  pore filters (Sartorius, Göttingen, West Germany), Hanks' balanced salt solution without phenol red (Microbiological Associates, Walkersville, Md.), ovalbumin recrystallized five times (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.), dextran and Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.), silica gel H 250- $\mu\text{m}$  layer plates (Analtech, Inc., Newark, Del.), arachidonic acid (Supelco, Inc., Bellefonte, Pa.), [5,6,8,9,11,12,14,15- $^3\text{H}$ (N)]arachidonic acid ([ $^3\text{H}$ ]arachidonic acid) sp act, 60 Ci/mmol, (New England Nuclear, Boston, Mass.), indomethacin (Sigma Chemical Co., St. Louis, Mo.), and 39-mm  $\times$  30-cm  $\mu\text{Porasil}$  columns (Waters Associates, Inc., Milford, Mass.) were obtained as noted. All organic solvents had been distilled from glass (Burdick & Jackson Laboratories Inc., Muskegon, Mich.). 5,8,11,14-eicosatetraenoic acid (ETYA) was supplied by Dr. James G. Hamilton (Hoffmann-La Roche, Inc., Nutley, N. J.).

*Purification and Characterization of the HETE Products of Human Neutrophil Lipoxygenase.* Human neutrophils were harvested from venous blood of normal subjects and enriched by centrifugation on Ficoll-Hypaque cushions to a purity of >96% as described (5, 6). The purified neutrophils were suspended at a concentration of  $4 \times 10^8/\text{ml}$  in 0.34 M sucrose buffered with 0.02 M Tris-HCl (pH 7.4) and containing 200  $\mu\text{M}$  indomethacin, and were homogenized in a glass tube with a Teflon pestle (Tri-R Instruments, Inc., Rockville Centre, N. Y.) at 4°C. The homogenate was centrifuged at 17,000 g for 20 min at 4°C. The supernate was removed and the pellet was

resuspended in the same volume of 0.34 M sucrose-Tris-HCl containing indomethacin. The resuspended pellet was sonified for 2 min at 4°C with intermittent pulses of 200 W (model W140D, Branson Sonic Power Co., Danbury, Conn.). The supernate and sonified pellet were dialyzed for 16 h at 4°C against 0.1 M  $\text{KH}_2\text{PO}_4$  containing 0.06 M sodium acetate and 200  $\mu\text{M}$  indomethacin (pH 6.0). The dialyzed preparations were emulsified with 2 mg of arachidonic acid and  $2 \times 10^6$  dpm of [ $^3\text{H}$ ]arachidonic acid and were incubated at 37°C for 6 h. The mixtures then were acidified and extracted as described (5-7) and the extracts were evaporated to dryness under  $\text{N}_2$  and redissolved in 100  $\mu\text{l}$  of methanol.

The methanol solution of arachidonic acid metabolites was applied to a silica gel H plate that was developed in ascending fashion in hexane:ethyl acetate:glacial acetic acid (1:1:0.005, vol:vol:vol). A mixture of HETE products was recovered from 1-cm strips of the silica gel plate by elution with three portions of 2 ml of chloroform:methanol (2:1, vol:vol). The eluates were pooled, evaporated to dryness under  $\text{N}_2$ , redissolved in 150  $\mu\text{l}$  of hexane:glacial acetic acid (125:1, vol:vol; solvent A) and injected onto a  $\mu\text{Porasil}$  column that was maintained at a flow rate of 1 ml/min by a gradient high-pressure liquid chromatography (HPLC) system (model No. 322, Altex Scientific Inc., Berkeley, Calif.). The solvent program consisted of isocratic elution with solvent A for 10 min followed by a linear gradient from solvent A to chloroform:methanol:glacial acetic acid (125:5:1, vol:vol:vol; solvent B) for 120 min. The optical density of the column effluent was monitored continuously at 240 nm with a Hitachi model 100-40 spectrophotometer (Hitachi Ltd., Tokyo, Japan) equipped with an Altex flow cell (Altex Scientific Inc.) and a Hewlett-Packard model 3380A integrator-recorder (Hewlett-Packard Co., Palo Alto, Calif.). Portions of each 1-ml fraction were assessed for radioactivity, optical density at 235 nm, and chemotactic activity for human neutrophils. The compounds in each fraction with radioactivity and neutrophil chemotactic activity were esterified with ethereal diazomethane and the methyl esters were transformed to trimethylsilyl derivatives (5-7). The  $\delta$ -lactone of 5-HETE was converted to the free acid by incubation in aqueous  $\text{Na}_2\text{CO}_3$  (2 g/100 ml) for 16 h at room temperature, and the 5-HETE was recovered immediately before methylation by titration of the solution to pH 3.0 with 0.1 M  $\text{H}_3\text{PO}_4$  and extraction with diethyl ether. Gas chromatography was carried out with simultaneous measurement of mass and radioactivity by employing a Varian 2700 instrument (Varian Associates, Instrument Div., Palo Alto, Calif.) combined with a Packard 894 gas proportional counter (Packard Instrument Co., Inc., Downers Grove, Ill.); the 6-ft column was packed with 1% SE-30 Ultraphase (Pierce Chemical Co., Rockford, Ill.) on Chromosorb-W (Johns-Manville, Filtration & Indus. Mineral Div., Denver, Colo. [high purity]) and was maintained at 200°C. Mass spectra were recorded with an LKB 9000 system (LKB Instruments, Inc., Rockville, Md.) equipped with an identical column; helium flow was 40 ml/min, the flash heater and separator were at 240°C, and ionizing energy was 22.5 eV. Purified HETE(s) were quantitated by determining the optical density of methanolic solutions at 235 nm; the concentration was calculated by employing a molar extinction coeff of 23,500 that had been derived from a standard methanolic solution of highly purified 12-L-HETE derived from platelets (5, 8).

*Assessment of In Vitro Neutrophil Chemotactic Activity.* Human neutrophils were harvested and purified as noted above, and were washed and resuspended at a concentration of  $4.2 \pm 0.2 \times 10^6$ /ml in Hank's balanced salt solution made 0.005 M in Tris-HCl, pH 7.4, containing 0.4 g/100 ml ovalbumin (HBSS-OA) (5, 6). Incubation conditions, processing of filters, and the enumeration of neutrophils in stained filters were as detailed (5, 6). Chemokinetic activity was assayed by utilizing an equal concentration of the HETE in the stimulus compartment and in the leukocyte well. Chemotactic and chemokinetic responses were expressed as net neutrophils/high-power field (hpf) after subtraction of the background migration found in chambers lacking a stimulus.

## Results

To determine the extent of conversion of [ $^3\text{H}$ ]arachidonic acid to HETE(s) by human neutrophil-derived lipoxygenase, extracts of incubation mixtures were chromatographed on silica gel H thin-layer plates in a solvent system that resolved arachidonic acid ( $R_f$ : 0.75-0.78) from 12-L-HETE ( $R_f$ : 0.53-0.59) (6). The ratio of the

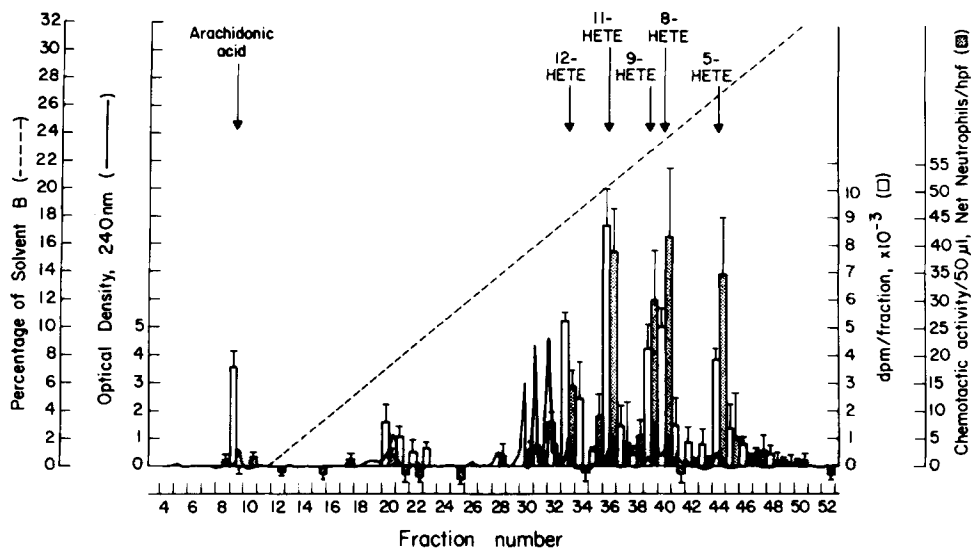


FIG. 1. High-pressure liquid chromatography of human neutrophil HETE(s). Each bar depicts the mean  $\pm$  one-half of the range of the radioactivity in each 1-ml fraction and of the chemotactic activity in 50- $\mu$ l portions for three consecutive preparations of HETE(s) generated by the 17,000-*g* supernates from homogenates of  $7.4$ ,  $6.1$ , and  $8.8 \times 10^8$  neutrophils, respectively. The background neutrophil migration in the absence of a stimulus was 4.7, 6.2, and 6.8 neutrophils/hpf for the three chemotactic assays. The optical density pattern represents one typical chromatogram.

radioactivity associated with the HETE(s) ( $R_f$ : 0.38–0.60) to the total radioactivity recovered from the plate provided an assessment of the percentage of conversion of the [ $^3$ H]arachidonic acid by the neutrophil lipoxygenase. The 17,000-*g* supernates from homogenates of neutrophils obtained from six consecutive normal donors converted a mean of 16.1% (range: 12.4–23.7%) of the [ $^3$ H]arachidonic acid to HETE(s) in 6 h at pH 6.0 and 37°C. The sonicated 17,000-*g* pellet from the corresponding neutrophil homogenates converted a mean of 5.7% (range: 2.9–9.8%) of the [ $^3$ H]arachidonic acid to HETE(s) under the same conditions. The mean extent of conversion of [ $^3$ H]arachidonic acid to HETE(s) by three of the 17,000-*g* supernates fell from 14.8% at pH 6.0, to 8.3% at pH 8.0, to 3.7% when the supernate was heated at 100°C for 20 min before the addition of [ $^3$ H]arachidonic acid, and to 4.5% in the presence of 100  $\mu$ M ETYA.

The mixture of HETE(s) from the silica gel H plate was subjected to  $\mu$ Porasil column HPLC (Fig. 1). Traces of residual arachidonic acid, as well as other contaminants, were separated from five discrete peaks of optical density, radioactivity, and chemotactic activity which eluted between 17 and 28% solvent B. Five mono-HETE(s) were identified by gas chromatography and mass spectrometry analysis of methyl ester-trimethylsilyl derivatives. All of the HETE(s) exhibited the same retention time as assessed by determinations of mass and radioactivity. 12-HETE in fraction 33, 11-HETE in fraction 36, and 5-HETE in fraction 44 were conclusively identified by comparing the mass spectra with those that had been obtained previously (1, 2, 7). The presence of 9-HETE in fraction 39 was established by the finding of abundant ions at  $m/e$  255 and 151, which indicated a preferential cleavage at the C9–C10 bond, as well as ions at  $m/e$  391 (M-15), 375, 331, 223, 181, and 165. The presence of 8-

HETE in fraction 40 similarly was established by the detection of an abundant ion at  $m/e$  265, which indicated a cleavage at the C8-C9 bond, as well as ions at  $m/e$  406, 391, 375, 316, 295, 199, 175, and 129. Lesser quantities of radioactive compounds were found in fractions 51 and 55 of 2 of 6 preparations, and these generated mass spectra compatible with those of di-HETE(s). As assessed by optical density at 235 nm, the mean yield of purified mono-HETE(s) per  $10^8$  neutrophils for three consecutive preparations of lipoxygenase was 2.1  $\mu\text{g}$  of 12-HETE, 6.4  $\mu\text{g}$  of 11-HETE, 1.8  $\mu\text{g}$  of 9-HETE, 2.4  $\mu\text{g}$  of 8-HETE, and 3.5  $\mu\text{g}$  of 5-HETE. The  $\delta$ -lactone of 5-HETE is an acid-catalyzed conversion product of 5-HETE acid that exhibits reduced chemotactic activity relative to the free acid and elutes from  $\mu$ Porasil just before 9-HETE. When this product was recovered and quantitated by optical density, the actual total yield of 5-HETE per  $10^8$  neutrophils was 4.8  $\mu\text{g}$ .

The increasing ratio of chemotactic activity to radioactivity that was observed in the sequence of peaks from 12-HETE to 5-HETE (Fig. 1) suggested a progressive increase in the chemotactic potencies of the HETE(s). Dose-response studies demonstrated that the chemotactic activity of 5-HETE was appreciated at concentrations as low as 40 ng/ml and reached a plateau by 1  $\mu\text{g}/\text{ml}$ , as compared to 5  $\mu\text{g}/\text{ml}$  for 8-HETE and 9-HETE, and 10  $\mu\text{g}/\text{ml}$  for 11-HETE and 12-HETE (Fig. 2). The mean maximal chemotactic activities of the HETE(s) were not significantly different. The peak chemokinetic responses to the HETE(s) were manifested at concentrations of 0.2–2  $\mu\text{g}/\text{ml}$  and were of a lesser magnitude than the maximal chemotactic responses. 5-HETE exhibited the highest ratio of chemokinetic to chemotactic activity, eliciting peak chemokinetic responses of approximately one-half of the maximal chemotactic response.

### Discussion

The incubation of [ $^3\text{H}$ ]arachidonic acid with the lipoxygenase-containing 17,000- $g$  supernate from homogenates of human neutrophils leads to the generation of a series of mono-HETE(s) which differ in structural and functional characteristics. The apparent effective polarity of the lipoxygenase metabolites increases progressively from 12-HETE to 5-HETE which permitted the resolution by silica gel HPLC of five distinct principles, namely 5-HETE, 8-HETE, 9-HETE, 11-HETE, and 12-HETE (Fig. 1). As assessed by the optical density of the isolated HETE(s), 11-HETE and 5-HETE predominated quantitatively and were recovered in amounts that were two- to threefold greater than those of the other three principles. 8-HETE and 9-HETE are newly recognized arachidonic acid products of mammalian cells. Previous analyses of the products of rabbit PMN leukocyte lipoxygenase revealed only 5- $\text{D}$ -HETE from arachidonic acid and 8-OH-9,11,14-eicosatrienoic acid from homo- $\gamma$ -linolenic acid (2). That the HETE(s) were derived from arachidonic acid was verified by the concomitant recovery of radiolabel along with each peak exhibiting both the characteristic optical density at 235–240 nm (7) and chemotactic activity for neutrophils (Fig. 1). A specific neutrophil lipoxygenase was implicated in the production of the HETE(s) as the generation was eliminated by prior boiling of the neutrophil homogenate, was reduced at pH 8.0 as compared to pH 6.0, and was suppressed by the presence of ETYA, an inhibitor of lipoxygenase and cyclo-oxygenase activity (8). The demonstration by mass spectrometric analyses of the presence in the HETE(s) of a hydroxyl group  $\alpha$  to a pair of conjugated *cis/trans* double bonds further suggested the

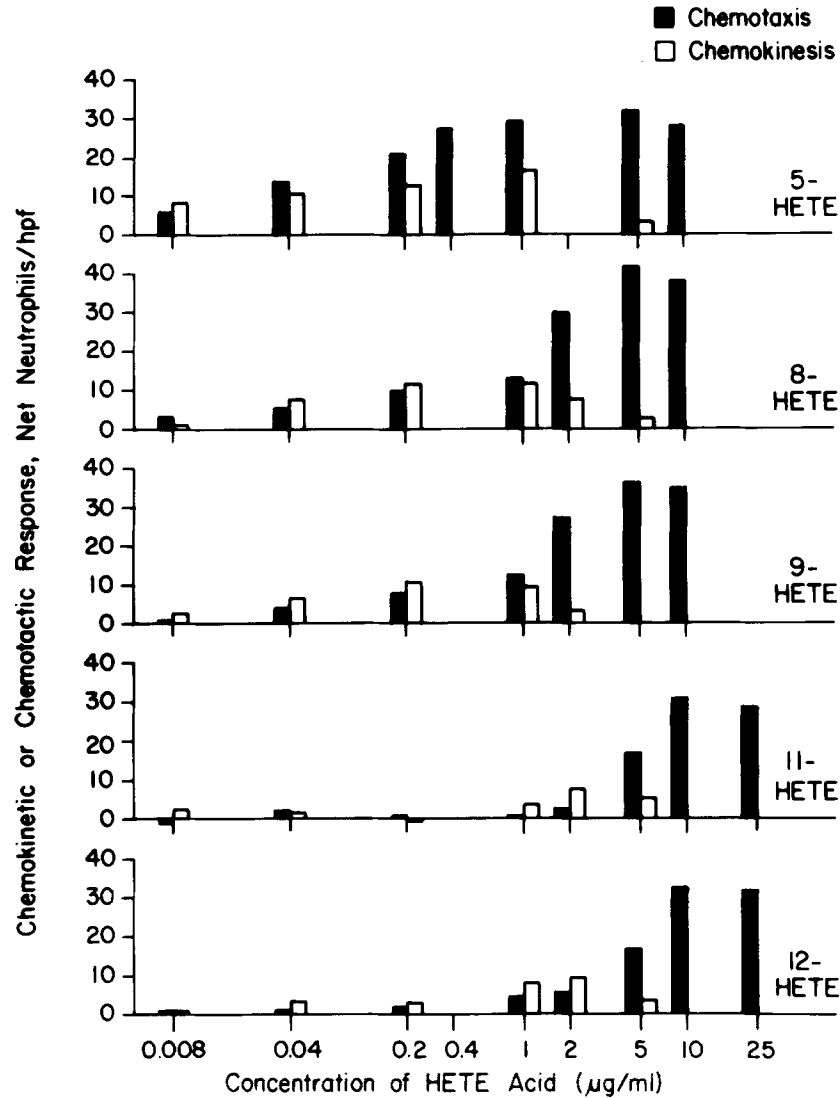


FIG. 2. Effects of endogenous HETE(s) on neutrophil migration. Each bar represents the mean of two experiments carried out in duplicate; background neutrophil migration in the absence of a stimulus was 6.1 and 4.3 neutrophils/hpf, respectively. The concentration of HETE indicated was present in the stimulus compartment in chambers employed to assess chemotaxis, and in both the stimulus and neutrophil compartments in chambers used to assess chemokinesis.

involvement of a lipoxygenase in the oxygenation of arachidonic acid. It is of interest that, as for the platelet lipoxygenase (9), the activity was found in the 17,000-g supernate that contains cytosol, microsomes, and other membrane fragments, but not in the 17,000-g pellet that contains the bulk of the nuclei, some aggregated membranes, and lysosomal granules (10).

The peak chemotactic responses to the endogenous HETE(s) were comparable in magnitude to that seen with platelet-derived 12-L-HETE (5, 6) and were achieved at concentrations of 1 µg/ml for 5-HETE, 5 µg/ml for 8-HETE and 9-HETE, and 10

$\mu\text{g/ml}$  for 11-HETE and 12-HETE (Fig. 2). In contrast to the differences in chemotactic potency of the HETE(s), all of the lipoxygenase products exhibited maximal chemokinetic activity at concentrations in the range of 0.2–2  $\mu\text{g/ml}$ . Of the neutrophil-derived purified HETE(s), 5-HETE was the most active chemokinetic factor. If the production of 5-HETE by intact neutrophils approaches the yield of  $\cong 50 \text{ ng}/10^6$  neutrophils that was realized for the solubilized lipoxygenase, then the endogenous 5-HETE would effect a 100–200% chemokinetic stimulation of the neutrophils (Fig. 2). Any increase in the production of 5-HETE by intact neutrophils as the result of stimulation of lipoxygenase activity by chemotactic factors or other inflammatory mediators would serve to increase further the level of chemokinetic stimulation.

### Summary

Incubation of [ $^3\text{H}$ ]arachidonic acid with the 17,000-g supernatant from homogenates of human neutrophils in the presence of indomethacin generated the unique metabolites 9-OH-5,7,11,14-eicosatetraenoic acid (9-HETE) and 8-HETE, in addition to 12-HETE, 11-HETE and 5-HETE. The human neutrophil chemotactic activity of the HETE products exhibited a rank-order of potency with 5-HETE  $\gg$  8-HETE = 9-HETE > 11-HETE = 12-HETE. The expression of chemokinetic activity as well as chemotactic activity suggested that the endogenous production of these principles may influence the mobility of human neutrophils.

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