POLYMORPHONUCLEAR LEUKOCYTE CHEMOTAXIS TOWARD
OXIDIZED LIPID COMPONENTS OF CELL MEMBRANES*

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The movement of polymorphonuclear leukocytes (PMN) toward a site of infection or injury is directed by a chemical gradient produced at the site, i.e., chemotaxis. Leukocyte chemotaxis has been extensively investigated using the Boyden technique (1, 2) and morphological methods (3, 4). Several biologically important peptides and proteins including the C5a fragment of complement, kallikrein, fibrinopeptide B, and transfer factor (5-8) possess chemotactic activity. Recently, we reported that lipids extracted from cotton and crude casein may be chemotactic in the absence of chemotactic proteins (9). In order to verify the existence of chemotactic lipids we have examined a series of pure fatty acids and related lipids.

This study presents evidence that lipids derived from the oxidation of arachidonic acid and similar polyenoic compounds can be true chemotaxins for PMN in the Boyden chamber assay system. These data suggest that oxidation of the abundant polyenoic fatty acids found in cell membranes as phospholipids and cholesteryl esters may be a source of chemotactic messages after cell injury.

Materials and Methods

Chemotaxis Assay All chemotaxis assays were performed in 4 mM sodium bicarbonate buffer medium, pH 7.4, containing 0.5% bovine serum albumin, 0.1% dextrose, and 0.85% sodium chloride. Lipids to be tested for chemotactic activity were dissolved in 98% ethanol. Samples of these solutions were placed in 15 × 100 mm glass culture tubes and the solvent was removed by evaporation under nitrogen. The residue was suspended in 1.8 ml of buffer medium. This solution was placed in the lower compartment (agent side) of a modified Boyden chamber (1, 10) while 0.8 ml of the same buffer containing 1.6 × 10⁶ PMN was added to the upper compartment (cell side). An 8 μm Sartorius filter, 13-mm diameter, (Science Essentials, Anaheim, Calif.) separated the solutions. Micropore filters of 1, 2, 5, and 8 μm porosity from the Millipore Corp. Bedford, Mass. and Nuclepore from Wallabs, San Rafael, Calif., were also used. After incubation at 37°C for 2 h 15 min, the solutions were removed and the filters were fixed with 70% ethanol and stained with hematoxylin (1). The cell side of the filter always consisted of a continuous monolayer of cells; thus, only agent side cell counts are reported here.

Isolation of PMN Leukocytes PMN cells were isolated from human blood collected in two 10 ml heparinized Vacutainers (Becton-Dickinson & Co., Rutherford, N. J.). The blood was mixed with 1.5 ml of sterile 0.85% NaCl containing 2% methylcellulose and allowed to separate for 45-60 min. The plasma layer was drawn off and centrifuged for 10 min at 180 g. The packed cells were suspended in 20 ml of 0.2% NaCl to lyse residual erythrocytes; then 20 ml of 1.5% NaCl was added. After centrifugation for 10 min at 180 g, the leukocyte pellet was suspended in buffer medium. The cell count was adjusted to 2 × 10⁶ cells/ml.

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Lipids for Chemotaxis Assays  Lipids purchased from Supelco, Inc., Bellefonte, Pa., Nu-Chek, Elysian, Minn., and Sigma Chemical Co., St. Louis, Mo. were checked for purity using thin-layer chromatography (Silica H; developed with chloroform, methanol, water, acetic acid; 65/35/1/1, vol/vol) and gas chromatography (elution of methyl esters from 3% OV-225, temperature gradient of 155-210°C/16 min). The following lipids were tested for chemotactic activity: (a) Fatty acids: caprylic, capric, lauric, myristic, palmitic, palmitoleic, anteisoheptanoic, stearic, α-hydroxystearic, oleic, linoleic, linolenic, arachidic, ε-5-eicosanoic, eicosatrienoic, arachidonic, eicosapentaenoic, brassidic, and docosahexaenoic acids. (b) Other lipids: prostaglandins E₂, E₃, A₄, and F₅ (gifts of Dr. J. E. Pike, Upjohn Co., Kalamazoo, Mich.); methyl arachidonate; ethyl arachidonate; arachidonyl acetate; arachidonoyl alcohol; sodium arachidonate; cholesteryllinolenate; and cholesterol.

Aerobic Oxidation and Ultraviolet Photolysis of Lipids  Solutions of lipids were applied to silica thin-layer chromatography plates (up to 200 μg lipid/cm² silica) and the solvent was allowed to evaporate. Oxidation of the lipids was effected by either of two methods with essentially the same results: (a) The thin-layer chromatography plate was exposed to air for 24 h at 20°C or (b) the plate was exposed to 265 nm radiation until maximal fluorescence developed in the lipid.

The oxidized lipid was extracted from silica using chloroform:methanol (2/1 vol/vol) and pure methanol. Suspended silica was removed by centrifugation and the solvent was evaporated under nitrogen.

Results

Chemotactic Activity of Pure Lipids  None of the fatty acids or lipids listed above were chemotactic in the Boyden chamber assay system. However, arachidonic acid from several sources (Supelco, Inc., Nu-Chek, and Sigma Chemical Co.) and eicosapentaenoic acid (Supelco, Inc.) elicited localized migration of PMN to produce round cell clusters on the agent side of the micropore filter (Fig. 1 A). We attributed this “clustering” behavior to the presence of small quantities of a chemotactically active oxidation product concentrated on the surface of arachidonic or eicosapentaenoic acid oil droplets. Consequently, we aerobically oxidized samples of arachidonic acid and all other unsaturated compounds listed in the Materials and Methods.

Chemotactic activity of oxidized lipids  The only compounds that produced significant chemotactic activity upon aerobic oxidation were arachidonic acid, eicosapentaenoic acid, linolenic acid, docosahexaenoic acid, and arachidonoyl acetate. As shown in Table I, arachidonic and eicosapentaenoic acids were nearly as chemotactic as Escherichia coli growth medium, while the other compounds were weakly chemotactic. Cell clusters were not observed in experiments using any of the oxidized lipids (e.g., Fig. 1 B).

Oxidized arachidonic acid usually lost all chemotactic activity when exposed to air for more than 5 days. Heating in air at 80°C for 10 min also destroyed the chemotactic properties of arachidonic acid. However, the chemotactic activity was stable for 24 h at 80°C in vacuo. Addition of 45 μg of oxidized arachidonic acid to the cell side of the Boyden chamber inhibited random PMN migration into the micropore filter, while 45 μg of autoxidized arachidonic acid added to both sides of the filter yielded no net chemotaxis of PMN.

Thin-layer Chromatography of Oxidized Arachidonic Acid  Aerobically oxidized arachidonic acid was analyzed on Silica H thin-layer chromatography using heptane-isopropyl ether-acetic acid (80/30/5, vol/vol) development. The material resolved into four iodine-positive bands at Rₜ 0.0, 0.20, 0.25, and 0.5. These bands were eluted and tested for chemotactic activity. Essentially all the chemotactic activity was in a zone from Rₜ 0.2-0.35 that did not seem to correlate with any of the iodine-positive bands. The material in this zone rechromato-
Fig. 1. PMN leukocytes that have migrated through an 8 μm Sartorius filter during a 2 h 15 min incubation at 37°C in response to arachidonic acid (A) and to aerobically oxidized arachidonic acid (B). The concentration of agent in each experiment was 56 μg/ml.
TABLE I
Chemotactic Activity of Oxidized Lipids

| Agent*                  | Concentration | Chemotaxis† |
|-------------------------|---------------|-------------|
| Arachidonic acid        | 56 µg/ml      | 120 ± 76    |
| Arachidonyl acetate     | 56            | 18 ± 9      |
| Eicosapentaenoic acid   | 56            | 125 ± 31    |
| Linolenic acid          | 56            | 22 ± 13     |
| Docosahexaenoic acid    | 56            | 19 ± 8      |
| Buffer medium           | 1 µl/l        | 1 ± 1       |
| E. coli growth medium   | 1:3 dilution  | 139 ± 53    |

*All lipids were subjected to aerobic oxidation before testing for chemotactic activity.
†Data from at least four experiments performed in duplicate using PMN cells from different individuals in each case. Five high power fields (hpf) were counted per duplicate.

graphed to $R_f$ 0.2-0.35 with retention of chemotactic activity. 1 mg of oxidized arachidonic acid yielded less than 100 µg of product in the zone $R_f$ 0.2-0.35 as estimated by gravimetric analysis.

Discussion
The present study indicates that pure lipids can act as chemotaxins for PMN in the absence of chemotactic peptides or proteins. As evidenced by thin-layer chromatography, the chemotactically active species in oxidized arachidonic acid is a small fraction of the total material. Thus, the chemotactic activity of oxidized arachidonic acid is expressed by doses of a few micrograms per milliliter. This activity is comparable to that reported for nitrogenous (protein) chemotaxins (5).

Mild oxidation is mandatory for the generation of chemotactic activity, at least in the case of arachidonic, eicosapentaenoic, docosahexaenoic, and linolenic acids, and arachidonyl acetate. More extensive oxidation results in a chemotactically inactive product. The prostaglandins listed above exhibited no chemotactic activity, even at concentrations as high as 100 µg/ml.

These data suggest that the high percentage of arachidonic acid in membrane phospholipids (11) and cholesteryl esters (12) represents a large reserve of precursor for chemotactic messages as well as for prostaglandins. It would only be necessary for the esterified arachidonic acid of a damaged membrane to be enzymatically oxidized (in situ or after lipolysis) to produce chemotaxins. Since lipid peroxidation occurs in tissues (11) it is possible that a lipid-derived chemotaxin-generating system operates in vivo. Continued oxidation of the active lipid would turn off the chemotactic message.

Summary
Polymorphonuclear leukocyte chemotaxis has been elicited by oxidized arachidonic acid and other oxidized polyenoic lipids in the Boyden micropore filter assay system. This chemotactic activity was observed in the absence of
serum and chemotactic proteins. The esterified arachidonic acid present in plasma membranes may be a precursor of chemotactic messages as well as prostaglandins in vivo.

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