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GENERATION OF A CHEMOTACTIC LIPID FROM ARACHIDONIC ACID BY EXPOSURE TO A SUPEROXIDE-GENERATING SYSTEM[1]

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Abstract—Certain products of arachidonic acid have been demonstrated recently to possess chemotactic activity for human polymorphonuclear leukocytes (PMN). Enzymatic (lipoxygenase, cyclooxygenase) generation of these lipid chemotaxins proceeds through the formation of intermediate lipid peroxides. Since lipid peroxidation can be mediated by oxygen-derived free radicals, we have examined whether chemotactically active products of arachidonic acid could be produced by exposing this unsaturated fatty acid to a superoxide-generating system. A lipid with potent chemotactic activity for human PMN was produced by incubating arachidonic acid with xanthine oxidase and acetaldehyde. Generation of chemotactic activity was time-dependent and could be inhibited to the greatest extent by scavengers of singlet oxygen (i.e., histidine, uric acid, and 2,5-dimethylfuran). Inhibition was also observed with scavengers of superoxide anion radicals (i.e., superoxide dismutase), hydrogen peroxide (i.e., catalase), and hydroxyl radicals (i.e., mannitol). Silica gel thin-layer radiochromatography demonstrated a single peak with chemotactic activity (R_f = 0.33–0.38) distinct from unaltered arachidonic acid. The product of arachidonic acid was chemotactic at a concentration of 3.0 ng/ml and chemokinetic at concentrations of 0.75–1.5 ng/ml. Since PMN produce oxygen-derived free radicals and singlet oxygen upon stimulation of their plasma membrane, and since arachidonic acid is widely distributed in human tissues, free radical-mediated generations of chemotactic activity from arachidonic acid may play an important role in amplifying inflammatory responses.

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INTRODUCTION

Two oxygenation products of arachidonic acid have been demonstrated recently to exhibit potent chemotactic activity for human polymorphonuclear leukocytes (PMN). One of these chemotaxins, 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) can be formed from arachidonic acid either by ultraviolet photolysis (1, 2) or by the action of platelet lipoxygenase (2, 3). The other chemotactic lipid, 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) is a product of the widely distributed fatty acid cyclooxygenase pathway (4). The action of cyclooxygenase on arachidonic acid also leads to the formation of stable prostaglandins, thromboxanes, and prostacyclin (5, 6).

HETE and HHT have been demonstrated to be chemotactic for human PMN in concentrations ranging from 0.7 to 25 μg/ml (3, 4). At very low concentrations, both compounds enhanced PMN migration in the absence of a concentration gradient, that is, they were positively chemokinetic (7). Low concentrations of these compounds also enhanced PMN migration in response to gradients of other chemoattractants. In these respects, HETE and HHT differ functionally from previously described peptide and protein chemotactic factors (8).

Enzymatic generation of HETE and HHT proceeds through the formation, respectively, of relatively unstable hydroperoxide and endoperoxide intermediates (9, 10). It is now appreciated that similar oxygenation products of lipids can be produced nonenzymatically by exposing unsaturated fatty acids to oxygen-derived free radicals (11–14). One such oxygen-derived free radical is the superoxide anion. Superoxide is formed as a consequence of the univalent reduction of molecular oxygen either by autooxidative processes or by enzymes involved in aerobic metabolism (15). Superoxide anion radicals not only react with each other (i.e., undergo spontaneous dismutation) to yield hydrogen peroxide and molecular oxygen, but also participate in a variety of reactions which yield even more highly reactive species (e.g., hydroxyl radicals and singlet oxygen) capable of mediating lipid peroxidation (11, 12, 15, 16).

Since oxygen-derived free radicals appear capable of reacting with unsaturated fatty acids to yield hydroperoxy and hydroxy compounds, we have examined the possibility that chemotactically active products might be formed by exposing arachidonic acid to a free radical-generating system. In this report, we present evidence that a potent chemoattractant for human PMN, with properties distinct from those of HETE and HHT, can be produced nonenzymatically from arachidonic acid by exposing this lipid to a superoxide-generating system.

MATERIALS AND METHODS

Superoxide-Generating System. Superoxide anion radicals were generated by the aerobic action of xanthine oxidase on acetaldehyde (11, 12). Reaction mixtures contained xanthine oxidase (0.12 units) (Sigma Chemical Co., St. Louis, Missouri) and acetaldehyde (24 μmol) (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, New York) in 1.0 ml of phosphate (10 mM)-buffered saline, pH 7.4 (Dulbecco's PBS, Grand Island Biological Co., Grand Island, New York). Generation of superoxide anion was quantified by measuring the reduction of ferricytochrome *c* (type III, Sigma Chemical Co.) as previously described (17). The amount of ferricytochrome *c* reduced per minute was calculated using an absorbance coefficient of 21.1/mM/cm at 550 nm (18).

Generation of a Chemotactic Product from Arachidonic Acid. Chromatographically pure arachidonic acid (0.5 mg) (Nu-Chek-Prep Co., Elysian, Minnesota.) was dissolved in absolute ethanol and incubated at 20°C with the superoxide-generating system. In some experiments, 0.3 μCi [^{14}C]arachidonic acid, specific activity 60.2 mCi/mmol (Amersham Corp., Arlington Heights, Illinois), was added to the reaction mixtures. Incubations were terminated by the addition of 10 ml chloroform–methanol (2:1, v/v) followed, after 20 min, by the addition of 2.0 ml 50 mM KCl (19). Incubation and extraction procedures were performed in the dark to avoid photooxidation of arachidonic acid (1–3). Organic solvents containing extracted lipids were evaporated at 42°C under nitrogen, and the residue obtained was used for both chemotactic and thin-layer chromatographic studies. The residue used for chemotactic studies was dissolved in absolute ethanol and added to phosphate (10 mM)-buffered 140 ml NaCl, pH 7.4, supplemented with 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 2.0% (w/v) bovine serum albumin (Grand Island Biological Co.). The final concentration of ethanol (0.005%, v/v) did not alter PMN viability or function. For thin-layer chromatography, the residue was dissolved in 1.0 ml chloroform-methanol (2:1).

Thin-Layer Radiochromatography. Thin-layer radiochromatography was performed on silica gel 60 plates (Brinkman Instruments Inc., Westbury, New York) which had been washed with methanol and activated for 30 min at 110°C. Samples were applied under nitrogen and chromatography was performed in the dark employing as a solvent system, chloroform—methanol–glacial acetic acid–water (65:35:1:1, v/v) containing butylated hydroxytoluene (0.05%, w/v) (Sigma Chemical Co.). Plates were dried under nitrogen, scanned for radioactivity (Packard Radiochromatogram Scanner, model 7230, Packard Instruments Co., Downers Grove, Illinois). and scraped in 0.5-cm sections. Scrapings were either added to 10 ml Bray's solution (20) for counting in a Beckman L7000 liquid scintillation counter (Beckman Instruments, Inc., Palo Alto, California) or subjected to extraction with chloroform-methanol (2:1). Extracted lipids were washed twice with 50 mM KCl to remove water-soluble contaminants and recovered from the organic phase after evaporation under nitrogen. Lipids were dissolved in absolute ethanol and diluted in buffer containing albumin.

Chemotaxis. PMNs were isolated from venous blood (32 ml) obtained from normal adult donors. Blood samples were allowed to sediment at room temperature after mixing with 8.0 ml acid-citrate dextrose (National Institutes of Health formula A) and 20 ml 6.0% dextran (average mol wt 234,000, Sigma Chemical Co.) in 140 mM NaCl. 35 ml of 0.67% NH_4Cl was added to 15-ml aliquots of the leukocyte-rich supernates, and the mixtures were centrifuged at 155*g* for 10 min. Pellets were washed once with 140 mM NaCl and finally suspended in phosphate (10 mM)-buffered 140 mM NaCl, pH 7.4, supplemented with 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 2.0% (w/v) bovine serum albumin. Cell suspensions contained approximately 85% PMN.

PMN random motility, stimulated random motility (chemokinesis), and directed migration (chemotaxis) were assessed by employing the "leading front" method of Zigmond and Hirsch (7). Aliquots (0.8 ml) of leukocyte suspensions (containing 2.5×10^6 PMN/ml) were added to the upper compartments of modified Boyden chambers (Nucleopore Corp., Pleasanton, California). These were separated from the lower compartments (containing buffer or dilutions of the chemoattractant) by 3.0 μm (pore diameter) cellulose nitrate micropore filters (Sartorius Filters, Inc., San Francisco, California). Chambers containing cells and chemoattractant were incubated at 37°C for 45 min in an atmosphere of 5% CO_2 and 100% humidity. The filters were then removed, fixed in methanol, stained with hematoxylin, dehydrated in ethanol, and cleared in xylene. The response of PMN either to buffer alone (random motility) or to various concentrations of the chemoattractant in the upper and/or lower compartments is reported as the distance the leading front of cells migrated into the filter (μm/45 min). Triplicate chambers were employed in each experiment and ten fields were examined in each filter.

Platelet Aggregation. Platelet-rich plasma (PRP) was prepared from venous blood anticoagulated with sodium citrate as described previously (21). Changes in light transmission were measured in a Payton aggregation module (Payton Associates, Buffalo, New York) after the addition of arachidonic acid (as the sodium salt), adenosine diphosphate (Sigma Chemical Co.) or 9,11-azoprostanoid III (22) (generously provided by Dr. E.J. Corey, Cambridge, Massachusetts). The minimum concentration of aggregating agent required for full aggregation was determined for each preparation of platelets and designated the threshold concentration. All studies were performed using threshold concentrations of the aggregating agents.

Other Compounds and Reagents. Bovine superoxide dismutase was obtained from Truett Laboratories, Dallas, Texas. Catalase, mannitol, histidine, uric acid, and 2,5-dimethylfuran were from Sigma Chemical Co. Highly purified [^{3}H]HETE was generously supplied by Dr. Edward Goetzl of Harvard Medical School. All solvents were either reagent grade (Eastman Kodak Co.) or Fisher-certified (Fisher Scientific Co., Pittsburgh, Pennsylvania).

RESULTS

Generation of Chemotactic Activity. The aerobic action of xanthine oxidase (0.12 units/ml) on acetaldehyde resulted in the generation of superoxide anion radicals (measured as superoxide dismutase-inhibitable reduction of ferricytochrome *c*) at an initial rate of 3.8–4.2 nmol/min (Figure 1). Generation of superoxide could be detected for at least 30 min, albeit at a rate which was not linear with respect to time. When chromatographically pure arachidonic acid was incubated with the xanthine oxidase–acetaldehyde system at 20°C for 30 min, chemotactic activity (see below) could be demonstrated in the extracted lipid fraction (Table 1). Chemotactic activity was generated only when arachidonic acid was incubated with the complete superoxide-generating system. None of the individual components of the incomplete system, either alone or in combination, were chemotactic. They failed to stimulate PMN migration beyond that observed with buffer alone.

Generation of chemotactic activity in this experimental system var-

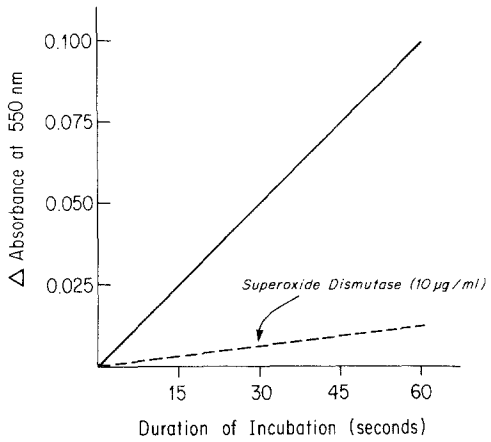

Fig. 1. Generation of superoxide anion radicals by the aerobic action of xanthine oxidase (0.12 units/ml) on acetaldehyde (24 mM) (measured as superoxide dismutase-inhibitable reduction of ferricytochrome *c*). Results are expressed as the change in absorbance at 550 nm versus time. Curves were traced from actual recordings.

ied with the duration of incubation and could be inhibited significantly by superoxide dismutase (Figure 2). Chemotactic activity could be detected as early as 5 min after the reaction was initiated and increased almost linearly with further incubation up to 30 min. Whereas these data are consistent with a role for superoxide anion radicals in the generation of chemotactic activity, they do not exclude participation by other reactive molecules (e.g., hydroxyl radicals and/or singlet oxygen) formed either as a conse-

Table 1. Generation of Chemotactic Activity from Arachidonic Acid by Exposure to Superoxide-Generating System

Reaction mixture[a]	PMN migration (μm/45 min)[b]
Buffer alone (random motility)	81.7 ± 3.5
Acetaldehyde (24 mM)	82.3 ± 3.0
Xanthine oxidase (0.12 units/ml)	83.6 ± 3.2
Arachidonic acid (0.5 mg/ml)	82.5 ± 3.9
Arachidonic acid + xanthine oxidase	81.8 ± 4.3
Arachidonic acid + acetaldehyde	82.7 ± 3.5
Xanthine oxidase + acetaldehyde	83.3 ± 3.2
Arachidonic acid + xanthine oxidase + acetaldehyde	100.9 ± 2.9[c]

[a] Reaction mixtures were incubated at 20°C for 30 min before addition of chloroform–methanol (2:1, v/v). Extracted lipids were dissolved in ethanol and diluted with buffer (10 ml) containing albumin.
[b] Results represent the mean ±*SEM* of 5 experiments.
[c] *P* versus buffer alone < 0.01 (Student's *t* test).

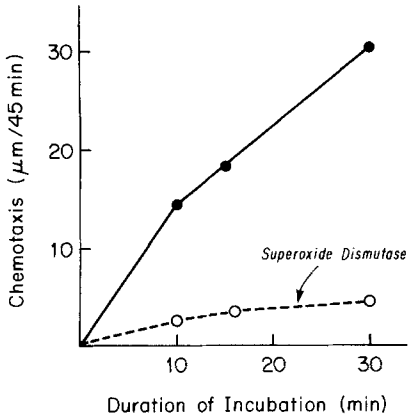

Fig. 2. Generation of chemotactic activity from arachidonic acid by exposure to xanthine oxidase and acetaldehyde. Effect of duration of incubation and superoxide dismutase (10 μg/ml). Results are expressed as net PMN migration (minus random motility) toward lipids extracted from reaction mixtures after 10, 20, and 30 min of incubation.

quence of the spontaneous dismutation of superoxide or by interactions between superoxide and hydrogen peroxide (11, 12). Consequently, we examined effects of various scavengers of these reactive molecules on the generation of chemotactic activity. Results of these experiments are summarized in Table 2.

Generation by the xanthine oxidase–acetaldehyde system of chemotactic activity derived from arachidonic acid was inhibited significantly by superoxide dismutase as well as by catalase, a scavenger of hydrogen peroxide. Bovine serum albumin, at identical concentrations, did not in-

Table 2. Effect of Scavengers on Generation of Chemotactic Activity from Arachidonic Acid

Additions to reaction mixtures[a]	(n)	PMN migration (μm/45 min)[b]
None	(5)	23.6 ± 2.7
Superoxide dismutase (10 μg/ml)	(4)	9.8 ± 1.7
Catalase (10 μg/ml)	(4)	8.5 ± 2.5
Superoxide dismutase + catalase	(3)	8.8 ± 3.9
Mannitol (100 mM)	(4)	11.8 ± 4.1
Histidine (0.5 mM)	(3)	3.6 ± 2.4
Uric acid (0.5 mM)	(3)	5.8 ± 1.0
2,5-Dimethylfuran (0.5 mM)	(3)	7.4 ± 1.8

[a]Reaction mixtures containing arachidonic acid, xanthine oxidase, and acetaldehyde were incubated at 20°C for 30 min before lipid extraction. Extracted lipids were dissolved in ethanol and diluted in 10 ml of buffer containing albumin.

[b]Values represent net migration (minus random motility). Mean ±*SEM*, *n* = number of experiments.

hibit production of chemotactic activity in this experimental system (data not shown). The similar inhibitory effects observed when superoxide dismutase and/or catalase were added to the reaction mixtures suggested that the production of chemotactic activity from arachidonic acid was dependent on the generation of superoxide but not necessarily mediated directly by this free radical. Indeed, mannitol, a scavenger of hydroxyl radicals (11, 12, 23) and scavengers of singlet oxygen, such as histidine, uric acid, and 2,5-dimethylfuran (11, 12, 24, 25), significantly inhibited the generation of chemotactic activity. With the exception of superoxide dismutase, none of these compounds inhibited production of superoxide by the xanthine oxidase–acetaldehyde system. Furthermore, as determined by thin-layer radiochromatography (see below), yields of chemotactic lipid were reduced by these scavengers in proportion to the extent that they inhibited generation of chemotactic activity in these experiments. Thus, there was no evidence that the scavengers influenced the chemotactic activity of the lipid product formed from arachidonic acid.

Thin-Layer Radiochromatography. Lipids extracted from reaction mixtures containing [^{14}C]arachidonic acid were subjected to thin-layer chromatography on silica gel 60. Plates were scanned for radioactivity and scraped in 0.5-cm sections for chemotactic studies. Two major peaks of radioactivity were detected (Figure 3). One, with an R_f of 0.7 and completely devoid of chemotactic activity, corresponded to native arachidonic acid. The other peak, with an R_f of 0.33–0.38, proved to be a potent chemoattractant for human PMN. Similar studies of reaction mixtures lacking

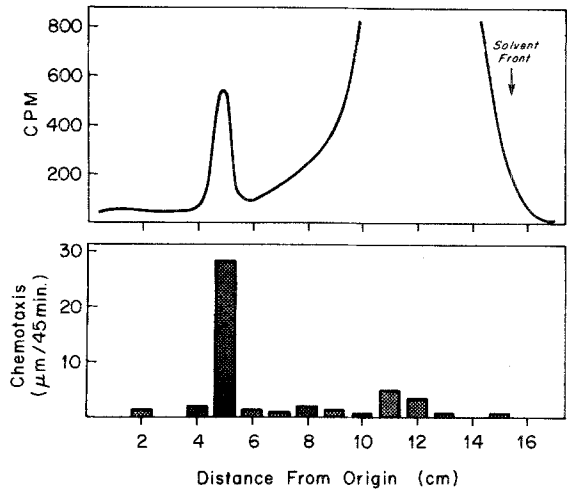

Fig. 3. Thin-layer radiochromatography and chemotactic activity of products of [^{14}C]arachidonic acid.

Table 3. Effect of Scavengers on Yield of Chemotactic Lipid Derived from Arachidonic Acid

Additions to reaction mixtures[a]	Yield of chemotactic lipid (μg)[b]
None	1.30
Superoxide dismutase (10 μg/ml)	0.80
Catalase (10 μg/ml)	0.70
Superoxide dismutase + catalase	0.75
Mannitol (100 mM)	0.60
Histidine (0.5 mM)	0.30
Uric acid (0.5 mM)	0.24
2,5-Dimethylfuran (0.5 mM)	0.11

[a]Reaction mixtures containing arachidonic acid (0.5 mg), xanthine oxidase, and acetaldehyde were incubated at 20°C for 30 min before lipid extraction and thin-layer chromatography.

[b]Yields of chemotactic lipid were calculated from the amount of radioactivity recovered from plates in sections with an R_f of 0.33–0.38. In reaction mixtures containing no scavengers, 0.25–0.27% of arachidonic acid was converted to the chemotactic lipid. Results represent average yields from two experiments.

either xanthine oxidase or acetaldehyde demonstrated only a single peak of radioactivity with an R_f of 0.7 and without chemotactic activity.

As indicated above, when scavengers of oxygen-derived free radicals or of singlet oxygen were added to reaction mixtures containing xanthine oxidase, acetaldehyde, and arachidonic acid, yields of the chemotactically active product with an R_f of 0.33–0.38 were reduced significantly (Table 3). There was a similar and significant reduction in the amount of chemotactic lipid generated when superoxide dismutase and/or catalase were added to the reaction mixtures. However, a more pronounced inhibitory effect was observed when scavengers of hydroxyl radicals and/or singlet oxygen were tested. Thus, there was a direct relation between the yield of lipid with an R_f of 0.33–0.38 and the ability of the various scavengers to inhibit the generation of chemotactic activity in whole reaction mixtures.

Radiochromatography of purified [^{3}H]HETE employing silica gel 60 and the previously described solvent system revealed this compound to have an R_f of 0.68. Thin-layer radiochromatography of photooxidized arachidonic acid (ultraviolet irradiation in air for 120 min) revealed two major peaks of radioactivity (not shown). One had an R_f of approximately 0.4 but was devoid of chemotactic activity (10–350 ng/ml) while the other had an R_f of approximately 0.7.

Thus, we were able to isolate a chemotactically active product, distinct from HETE, formed by the interaction of arachidonic acid with a free radical-generating-system. Radiochromatography enabled us to quantify the yield of this product and to examine some of its properties.

Properties of Chemotactic Lipid Derived from Arachidonic Acid. By adjusting the concentration of the chromatographically purified lipid added to the upper and/or lower compartments of the modified Boyden chambers, we were able to assess its relative chemokinetic and chemotactic activities (7). In this so-called "checker-board analysis," increased migration of cells in the absence of a concentration gradient (from upper to lower compartment) of the putative chemoattractant reflects stimulated random motility, or chemokinesis. On the other hand, enhanced migration of cells towards increasing concentrations of the lipid reflects directed motility, or chemotaxis. Our results (Table 4) indicate that the product formed from arachidonic acid exhibits chemokinetic activity at low concentrations (0.75–1.5 ng/ml), i.e., it enhanced PMN migration when added only to the cell compartments of the modified Boyden chambers or to both compartments at identical concentrations. Increasing amounts of lipid added to the cell compartments produced a concentration-related inhibition of random motility. When increasing amounts of lipid were added to the lower compartments, a concentration-related chemotactic response was observed. Chemotaxis was inhibited, however, when concentrations of lipid exceeding 3.0 ng/ml were simultaneously added to the cell compartments, an effect compatible with chemotactic "deactivation" (26). As shown in Figure 4, as little as 3.0 ng/ml exhibited significant chemotactic activity for human PMN. Maximum activity was observed at a concentration of approximately 24 ng/ml. Higher concentration resulted in increasing inhibition of cell migration and clumping of PMNs on the surfaces of the micropore filters.

Effect of Chemotactic Lipid on Platelet Aggregation. Addition of the isolated chemotactic lipid to normal platelet-rich plasma for 1–2 min

Table 4. Analysis of Chemokinetic and Chemotactic Activities of Lipid Derived from Arachidonic Acid

Concentration of lipid below filter (ng/ml)	PMN migration (μm/45 min)[a] concentration of lipid above filter (ng/ml)				
	0	1.5	3.0	6.0	12.0
0	99.7	106.1	103.4	93.1	92.8
1.5	102.1	104.5	99.5	94.8	92.7
3.0	111.4	102.0	99.2	99.1	98.3
6.0	120.0	112.1	105.0	101.0	97.4
12.0	127.2	116.0	103.0	97.5	95.5

[a]Results are expressed as PMN migration in response to buffer alone or various concentrations of lipid added to the cell compartment (above filter) and/or to the stimulus compartment (below filter) of modified Boyden chambers. Values represent the average of two experiments.

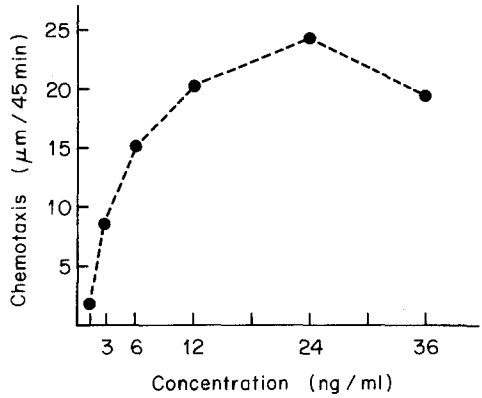

Fig. 4. Response of human PMN to the chemotactic lipid derived from aracidonic acid.

did not induce platelet shape change or aggregation (Figure 5). However, subsequent platelet aggregation responses to threshold concentrations of either sodium arachidonate or the endoperoxide analog, 9,11-azoprostanoid III (22), were delayed or inhibited, depending upon the amounts of chemotactic lipid added (Figure 5). Similarly, biphasic aggregation responses to adenosine diphosphate were delayed, and the second phase

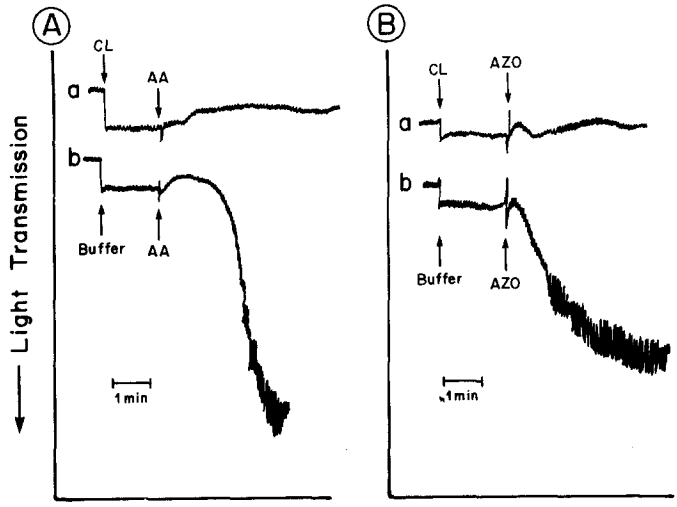

Fig. 5. Inhibition of platelet aggregation by the isolated chemotactic lipid (CL). Platelets were incubated either with CL or buffer for 90 seconds before the addition of the aggregation agents. A. (a) inhibition by CL (10 μg/ml) of arachidonic acid (AA)-induced aggregation; (b) effect of buffer. B. (a) inhibition by CL (20 μg/ml) of 9,11-azoprostanoid III (AZO)-induced aggregation; (b) effect of buffer.

of aggregation was inhibited by prior exposure of the platelets to the chemotactic lipid (not shown). Thrombin-induced aggregation of washed platelets also inhibited by the chemotactic lipid, with concomitant inhibition of [^{14}C]serotonin release (data not shown).

DISCUSSION

The results of the experiments described in this report can be interpreted as indicating conversion of arachidonic acid by the action of hydroxyl radicals and singlet oxygen to a product which possesses potent chemotactic activity for human PMN. Generation of chemotactic activity required incubation of arachidonic acid with an intact superoxide-generating system and could be inhibited significantly by superoxide dismutase and catalase, as well as by scavengers of hydroxyl radicals (i.e., mannitol) and singlet oxygen (i.e., histidine, uric acid, and 2,5-dimenthylfuran). It has been demonstrated previously that superoxide and hydrogen peroxide cooperate in generating hydroxyl radicals and singlet oxygen which oxidize unsaturated fatty acids (11, 12, 16). The chemotactically active product was extracted from reaction mixtures with organic solvents and behaved on silica gel 60 as a polar lipid. The yield of the chemotactically active product was reduced by the various scavengers in proportion to their ability to inhibit generation of chemotactic activity in whole reaction mixtures containing xanthine oxidase, acetaldehyde, and arachidonic acid. The inability of superoxide dismutase and catalase to completely suppress formation of the chemotactically active product could be explained by the susceptibility of these enzymes to inhibition by the large amounts of hydrogen peroxide generated in these reaction mixtures (27). Results of experiments employing histidine, uric acid, and 2,5-dimethylfuran, in which the greatest inhibition of generation of chemotactic activity was observed, suggest that singlet oxygen was largely responsible for the formation of the chemotactically active product.

The precise identity of the chemotactic lipid formed from arachidonic acid by exposure to a superoxide-generating system cannot be determined from the experiments described in this report. However, singlet oxygen is known to generate hydroperoxides from unsaturated fatty acids (16), and the chemotactic lipid proved to be more polar on thin-layer chromatography than either arachidonic acid or the hydroxy acid, HETE. Indeed, it recently has been demonstrated that singlet oxygen (generated by photolysis) converts arachidonic acid to biologically important hydroperoxides (28). These, as well as arachidonic acid dihydroperoxides, are capable of inhibiting platelet aggregation induced by adenosine diphosphate, epinephrine, and collagen (29, 30). Interestingly, monohydroxy and dihydroxy-

derivatives of arachidonic acid have been found to have no effect on platelet aggregation. Consequently, we suspect that the chemotactic lipid is a hydroperoxy acid or, possibly, a mixture of positional isomers of arachidonic acid hydroperoxides. Final identification, of course, must await analysis by gas chromatography and mass spectrometry. It can be stated with some certainty, however, that the chemotactic lipid described in this report is distinct from either HETE or HHT. It could be separated easily from highly purified [^{3}H]HETE by thin-layer chromatography and is chemotactically active at concentrations (3.0–36 ng/ml) which are considerably lower than those reported previously for HETE or HHT (0.7–25 μg/ml) (3, 4). In fact, the dose–response curve (Figure 4) very closely resembles that reported for the extremely potent, complement-derived chemoattractant, C5a (31). Nevertheless, the chemotactic lipid described here does share some properties of other oxygenation products of arachidonic acid. For example, it resembles HETE and HHT by exhibiting positive chemokinetic activity at concentrations less than those which are chemotactic. This property appears to be unique for lipid chemoattractants.

Peroxidation of unsaturated fatty acids after their exposure to simple free radical-generating systems has now been demonstrated by several investigators (11–13, 16). Kellogg and Fridovich (11), for example, demonstrated peroxidation of linolenate by the aerobic action of xanthine oxidase on acetaldehyde. Lipid peroxidation in this system was inhibited by superoxide dismutase and by catalase, indicating that both superoxide and hydrogen peroxide were essential intermediates. Peroxidation was also inhibited by scavengers of singlet oxygen and, to some extent, by scavengers of singlet oxygen and, to some extent, by scavengers of hydroxyl radicals. That singlet oxygen was a product of the reaction between xanthine oxidase and acetaldehyde was suggested by the ability of this system to convert 2,5-dimethylfuran to the same product which results from its exposure to a photochemical source of singlet oxygen. Conversion of dimethylfuran by the xanthine oxidase–acetaldehyde system was inhibited by superoxide dismutase and catalase, indicating that the production of singlet oxygen was dependent on interactions between superoxide and the product of its spontaneous dismutation, hydrogen peroxide.

More recent studies by Kellogg and Fridovich (12) have demonstrated that peroxidation of lipids by the aerobic action of xanthine oxidase on acetaldehyde could be inhibited significantly by 0.5 mM hypoxanthine, xanthine, and uric acid. These compounds were also found capable of inhibiting lipid peroxidation by a photochemical system, and it was therefore concluded that they were capable of scavenging singlet oxygen. This would provide an explanation for the difficulty other investigators have encountered in their attempts to demonstrate peroxidation of lipids due to

the aerobic action of xanthine oxidase on either xanthine or hypoxanthine. Whereas this system is a good source of superoxide anion radicals (32) and hydroxyl radicals (33), reactive singlet oxygen would be effectively scavenged not only by the purine substrates but also by product (i.e., uric acid).

It must be pointed out that there is no general agreement regarding the manner in which hydroxyl radicals and/or singlet oxygen are formed by superoxide-generating systems (34), nor is it clear how these reactive molecules interact with unsaturated lipids (11–13, 16). One recent report, for example, has suggested that spontaneous formation of lipid hydroperoxides is a prerequisite for further free radical-mediated lipid peroxidation (13). Although the precise mechanisms whereby free radicals induce lipid peroxidation remain controversial, there is little doubt that unsaturated fatty acids can be altered nonenzymatically when exposed to simple free radical-generating systems.

Valone and Goetzl (35) recently demonstrated that antigen challenge of the rat peritoneal cavity after passive preparation with IgG-rich hyperimmune antiserum released arachidonic acid-derived lipids that enhanced migration of human PMN. Both chemotactic and chemokinetic factors were isolated. It was suggested that these factors might act synergistically to promote a sustained influx of leukocytes to the site of immunologically-induced inflammation despite a decreasing concentration gradient of chemoattractants. The arachidonic acid-derived chemoattractant described in this report may similarly play an important role in amplifying inflammatory responses. Arachidonic acid is a ubiquitous constituent of cell membrane phospholipids and can be made accessible by the action of phospholipases to a variety of chemical transformations in response to membrane perturbations (36, 37). Phagocytic leukocytes (particularly PMN) generate abundant amounts of oxygen-derived free radicals and singlet oxygen upon stimulation of their plasma membrane (17, 38) and have been demonstrated previously to be capable of producing oxygenation products of arachidonic acid (39-42) and other lipid peroxides (43). It is of interest in this regard that Smolen and Shohet (44) were able to demonstrate a marked decrease during phagocytosis of the arachidonic acid content of phospholipids in the membranes of phagocytic vacuoles isolated from human PMN. Consequently, under conditions which exist at most foci of inflammation, it is quite possible that potent chemoattractants can be generated from arachidonic acid by mechanisms involving free radicals.

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