

LEUKOTRIENE GENERATION BY EOSINOPHILS*

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Leukotrienes are a novel group of chemical mediators generated by the oxidation of arachidonic acid by the lipoxygenase pathway (for reviews see 1, 2). One chemical pathway of leukotriene synthesis from arachidonic acid results in the formation of dihydroxy derivatives, which include leukotriene B₄ (LTB₄; 5-(S), 12-(R)-dihydroxy-6-*cis*-8,10-*trans*-14-*cis*-eicosatetraenoic acid)¹ (3) and its isomers (5-(S), 12-(R)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid (abbreviated 5-(S), 12-(R)-6-*trans*-LTB₄) and 5-(S), 12-(S)-dihydroxy-6,8,10-*trans*-14-*cis*-eicosatetraenoic acid (abbreviated 5-(S), 12-(S)-6-*trans*-LTB₄) (4). LTB₄ has potent chemotactic and chemokinetic activity for leukocytes (5, 6); in addition, recent evidence suggests that it induces the release of thromboxanes and prostaglandins from pulmonary tissue (7).

A second pathway of leukotriene synthesis results in the formation of agents that cause increased vascular permeability and smooth muscle contraction (8–10). These agents, called slow reacting substance (SRS), are generated in immediate hypersensitivity reactions and are thought to play an important role in asthma through their potent bronchiolar smooth muscle contracting activity. The first SRS that was structurally identified was leukotriene C₄ (LTC₄; 5-(S)-hydroxy-6-(R)-S-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) (11–13). The removal of glutamic acid from LTC₄ by γ -glutamyl transpeptidase results in the formation of the cysteinylglycine derivative leukotriene D₄ (LTD₄; 5-(S)-hydroxy-6-(R)-S-cysteinylglycine-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid), which also has potent SRS activity (14–17). Leukotriene E₄ (LTE₄; 5-(S)-hydroxy-6-(R)-S-cysteinyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) is formed on the removal of glycine from LTD₄ through the action of an aminopeptidase (16, 17). Stereoisomers of LTC₄ and LTD₄ that possess 11-*trans* double

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¹ Abbreviations used in this paper: β -NADH, β -nicotinamide adenine dinucleotide; ETYA, 5,8,11,14-eicosatetraenoic acid; HPLC, high-pressure liquid chromatography; LDH, lactate dehydrogenase; LTA₄, leukotriene A₄; LTB₄, leukotriene B₄; LTC₄, leukotriene C₄; LTD₄, leukotriene D₄; LTE₄, leukotriene E₄; SRS, slow reacting substance.

bonds (11-*trans*-LTC₄ and 11-*trans*-LTD₄, respectively) are also formed (1, 18). The SRS activity of biological fluids is due predominantly to LTC₄ and LTD₄ (1, 2).

Leukotriene generation by mastocytoma cells (11), basophils (14, 15, 17), macrophages (19–22), neutrophils (3, 4), and human lung tissue (23) has been described; their formation by eosinophils, an important participant in immediate hypersensitivity reactions, however, has not been previously reported. We report here that horse eosinophils stimulated with the calcium ionophore A23187 generate LTB₄, 5-(*S*), 12-(*R*)-6-*trans*-LTB₄, 5-(*S*), 12-(*S*)-6-*trans*-LTB₄, LTC₄, 11-*trans*-LTC₄, and LTD₄.

Materials and Methods

Special Reagents. Leukotriene standards were obtained as follows. LTB₄, 5-(*S*), 12-(*R*)-6-*trans*-LTB₄ and 5-(*S*), 12-(*S*)-6-*trans*-LTB₄ were isolated from A23187-stimulated human neutrophils (3, 4); LTC₄, 11-*trans*-LTC₄ and LTD₄ were isolated from A23187-stimulated mouse mastocytoma cells (11, 15); synthetic LTC₄ and LTD₄ were kindly provided by Dr. J. Rokach, Merck Frosst Laboratories, Pointe-Claire/Dorval, Quebec (24); ³H-LTC₄ from mouse peritoneal macrophages was the gift of Dr. C. A. Rouzer and Dr. W. A. Scott, The Rockefeller University, New York (21).

The SRS antagonist FPL 55712 was kindly provided by P. Sheard, Fisons Limited, Pharmaceutical Division, Loughborough, England; 5,8,11,14-eicosatetraenoic acid (ETYA) was provided by Dr. W. E. Scott, Hoffmann-LaRoche, Inc., Nutley, N. J.; and the calcium ionophore A23187 was provided by Dr. J. Hosley, Lilly Research Laboratories, Indianapolis, Ind. Arachidonic acid, soybean lipoxygenase (type I), histamine diphosphate, indomethacin, β-nicotinamide adenine dinucleotide, reduced form (β-NADH), pyruvic acid (sodium salt, type II), lactate dehydrogenase (LDH), and polyvinylpyrrolidone (40,000 mol wt) were obtained from Sigma Chemical Co., St. Louis, Mo., and Amberlite XAD-7 was obtained from Mallinckrodt Chemical Works, St. Louis, Mo. [1-¹⁴C]arachidonic acid (>50 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, Ill.

Isolation of Eosinophils. Eosinophils were isolated from 10–40 liters of horse blood (kindly provided by the Florence Packing Co., Florence, Wash.), as previously described (25). In brief, after 1 h of spontaneous sedimentation, the leukocyte-rich plasma and the upper quarter of the erythrocyte sediment were collected and centrifuged at 3,500 *g* for 15 min at 20°C. The eosinophils penetrated into the erythrocyte pellet during this centrifugation, whereas the remaining leukocytes formed a buffy coat layer at the cell-plasma interface. The buffy coat and platelet-containing plasma were removed by aspiration. Polyvinylpyrrolidone sedimentation followed by hypotonic lysis enabled a complete separation of the erythrocytes from the eosinophils. The eosinophils were washed with 1% NaCl and resuspended at a concentration of 5 × 10⁷ cells/ml in a standard salt solution, pH 7.2, consisting of 154 mM NaCl, 2.7 mM KCl, 4 mM Na₂HPO₄, 2.7 mM KH₂PO₄, 0.9 mM CaCl₂, 0.8 mM MgSO₄, and 6 mM glucose. The cell preparations consisted of 98–100% eosinophils (<2% neutrophils), which were >98% viable as measured by trypan blue exclusion.

Isolation of Neutrophils. Neutrophils were isolated from horse blood as follows. 30 ml of a 1:3 blood: 0.9% NaCl mixture was layered over 12 ml of Hypaque-Ficoll (specific gravity 1.0775) and the tubes centrifuged at 1,000 *g* for 40 min at 20°C. The upper layer, containing mononuclear cells, was removed by aspiration. The lower layer, containing polymorphonuclear leukocytes and erythrocytes, was mixed with 3% PVP (in 0.9% NaCl) and platelet-free horse plasma in a 3:2:1 mixture and allowed to sediment for 40 min at 4°C. The upper layer, containing neutrophils, was aspirated, and the cells were collected by centrifugation. Remaining erythrocytes were removed by hypotonic lysis, and the neutrophils were suspended in the standard salt solution. The neutrophil preparations were of >98% purity (1–2% eosinophils) and were >97% viable by trypan blue exclusion.

SRS and LDH Release. Duplicate samples of eosinophils or neutrophils were preincubated for 5 min at 37°C in standard salt solution, pH 7.2, in the presence and absence of arachidonic acid or inhibitors of arachidonic acid metabolism (see legends to figures and tables) in a water bath oscillating 80 times per min. The ionophore A23187 was then added to make a total

volume of 1.0 ml. Unless otherwise indicated, incubations were for 15 min at 37°C, after which the suspensions were placed on ice until centrifugation at 400 *g* for 6 min at 4°C. The supernatants and pellets were stored at -70°C until bioassay of SRS activity or measurement of LDH activity.

SRS Bioassay. SRS activity was assayed on the atropinized, antihistamine-treated guinea pig ileum, as previously described (26), using the model ABAC-100, Automatic Bio-Assay Controller, (ADAPS, Inc., Dedham, Mass.). 1 unit of SRS is defined as the concentration required to produce an ileal contraction equal in amplitude to that produced by 5.0 ng/ml histamine base. Peak contractions were measured 2 min after addition of the samples. Varying concentrations of FPL 55712 were added to the 7.0-ml bioassay chamber to test for inhibition of SRS-induced contractions (27). The reaction mixture was diluted at least 200-fold before application in the guinea pig ileal bioassay. None of the individual reaction mixture components (e.g., A23187, indomethacin, ETYA) caused ileal contraction or affected the contraction induced by synthetic LTC₄ or LTD₄ under the conditions used.

Measurement of LDH Release. LDH was measured using β-NADH as substrate, as previously described (28).

Purification of Eosinophil Leukotrienes. Column Chromatography. Eosinophils (3×10^8 in 27 ml of the standard salt solution) were preincubated for 5 min at 37°C in a shaking water bath. The ionophore A23187 (10 μg/ml, final concentration) was then added to make a total reaction volume of 30 ml and a final cell concentration of 10^7 cells/ml. After 15 min, the cells were pelleted at 400 *g* for 10 min at 4°C and discarded. The cell-free supernatant was adjusted to 80% ethanol (vol/vol). After storage for 16–20 h at 4°C, the ethanol suspension was centrifuged at 3,000 *g* for 10 min at 4°C, and the pellet was discarded. The clear supernatant was evaporated to dryness *in vacuo* at 30–45°C in a Buchler flash evaporator (Buchler Instruments Division, Searle Analytic, Inc., Fort Lee, N. J.). The SRS-containing residue was resuspended in 5.0 ml of 0.1 M NaOH, incubated for 30 min at 37°C, adjusted to pH 5.0, and applied over 60 min to an Amberlite XAD-7 column (1.0 × 30.0 cm), previously washed with 1 liter of acetone and 1 liter of distilled water. The column was washed with 300 ml distilled water, and elution was performed with 300 ml of 100% ethanol. The ethanol eluant was evaporated *in vacuo* and stored at -70°C (26).

In some experiments, eosinophils were preincubated with radiolabeled arachidonic acid. [¹⁴C]arachidonic acid (150 μg; 24 μCi) in a total volume of 0.9 ml was evaporated to dryness, redissolved in 0.1 ml ethanol, and added to the eosinophil preparation. After preincubation for 30 min at 37°C, A23187 was added, and the sample was treated as described above.

Reverse-Phase High-Pressure Liquid Chromatography (HPLC). The dried eluant from the Amberlite XAD-7 column was resuspended in 300 μl of methanol/water (65:35, vol/vol), containing 0.1% acetic acid (final pH, 3.9), centrifuged at 3,000 *g* for 15 min at 20°C, and the clear supernatant applied to a 3.9 × 300-mm μBondapak C₁₈ column (10 μm particle size) (Waters Assoc., Milford, Mass.). Elution was with the same solvent at a flow rate of 1.0 ml/min and a pressure of 1,600 P.S.I., using either a Waters model ALC 244 or model 334 MP (Beckman Instruments, Inc., Berkeley, Calif.) HPLC apparatus. The optical density of the column eluant was continuously recorded at 280 nm, and sequential 1-ml fractions were collected for assay of SRS activity. After 40 min of isocratic elution, a linear methanol gradient from 65 to 100% was applied to the column to remove all absorbed lipids. Biologically active fractions were evaporated to dryness under nitrogen and resuspended in a small volume of the methanol/water/acetic acid solvent. To obtain chromatographically pure compounds, each sample was rechromatographed at least twice, either as described above or on a 4.6 × 250-mm Ultrasphere ODS C₁₈ column (5 μm particle size; Altex Scientific Co., Berkeley, Calif.) with a mobile phase of methanol/water (72:28, vol/vol) with 0.1% acetic acid, pH 3.9, and a flow rate of 1 ml/min.

For identification, the samples underwent additional rechromatography on each of the two columns described above and on a 4.6 × 250-mm Nucleosil C₁₈, 5 μm particle size column (Machery-Nagel, Düren, West Germany) with a methanol/water (65:35, vol/vol) 0.01% acetic acid, pH 5.7, mobile phase and a flow rate of 1.5 ml/min, and their retention times were compared to those of appropriate standards.

Ultraviolet Spectrometry. The UV spectra of the rechromatographed compounds were determined using a Cary 219 spectrophotometer (Cary Instruments, Monrovia, Calif.). In some

experiments the measurements were made before and after incubation of the compound in the standard salt solution with soybean lipoxygenase (10 $\mu\text{g}/\text{ml}$) for 30 min at 20°C (11).

Gas Chromatography-Mass Spectrometry. Compounds that co-eluted on HPLC with the LTB₄, 5-(*S*), 12-(*R*)-6-*trans*-LTB₄ and 5-(*S*), 12-(*S*)-6-*trans*-LTB₄ standards were derivatized by methylation with diazomethane and trimethylsilylation with bis(trimethyl)trifluoroacetamide. Derivatives were analyzed by gas chromatography-mass spectrometry using a Finnigan 3200 quadrupole mass spectrometer (Finnigan Corp., Sunnyvale, Calif.) under electron impact (70 eV) conditions. Gas chromatographic separation was performed using 3% OV-101 on Supelcoport (140–160 mesh) in a glass column 0.2 \times 150-cm and temperature programming 240° to 280° at 8°/min. Equivalent chain length was determined by coinjecting reference fatty acid methyl esters, as previously described (29).

Statistical Analysis. The data are reported as the mean \pm SE of the combined experiments. Differences were analyzed for significance using Student's two-tailed *t* test for independent means (not significant, *P* > 0.05).

Results

Incubation of eosinophils with the calcium ionophore A23187 results in the release into the extracellular medium of an agent or agents that induces contraction of the guinea pig ileum. The characteristics of that contraction are those of SRS—a slow, sustained contraction in the presence of atropine and an antihistamine. Further, contraction was inhibited by the SRS antagonist FPL 55712. The concentration necessary to produce a 50% reduction of the guinea pig ileal contractile response (IC₅₀) was ~11 ng/ml (Table I), which is consistent with the concentrations of FPL 55712 reported to block 50% of the activity of the SRS obtained from guinea pig lung (27), rat basophilic leukemia cells (30), and rat peritoneal mast cells (31). Other smooth muscle stimulants, such as prostaglandins, serotonin, and bradykinin, are considerably less sensitive to FPL 55712: the IC₅₀ for these stimulants is 1,000 times greater than that for SRS (27). This suggests that most if not all of the smooth muscle contracting activity released from eosinophils by A23187 is due to SRS.

SRS production by eosinophils (5×10^6 cells/ml) increased with A23187 concentration to reach a maximum at 10 $\mu\text{g}/\text{ml}$ (1.9×10^{-5} M) (Fig. 1) with LDH release <5% at these concentrations. When the ionophore concentration was raised to 20 $\mu\text{g}/\text{ml}$ (3.8×10^{-5} M), SRS formation decreased and LDH release increased, suggesting cytotoxic injury to the cells. Unless otherwise indicated, the ionophore concentration used was 10 $\mu\text{g}/\text{ml}$. SRS release was evident 1 min after the addition of ionophore and increased with time to reach a maximum at 15–30 min (data not shown). Incubation for 15 min at 37°C was routinely used.

TABLE I
Inhibition of Eosinophil SRS Activity by FPL 55712

FPL 55712 <i>ng/ml</i>	Inhibition of SRS activity %
7	34.7 \pm 5.6 (5)
14	62.3 \pm 4.1 (5)
28	73.9 \pm 3.3 (3)

Supernatants from ionophore-treated eosinophils were diluted with standard salt solution to yield 1–2 units of ileal contracting activity per ml. FPL 55712 was added at the final concentrations indicated, and the percent inhibition of gut contraction was determined. The results are the mean \pm SE of (*n*) experiments.

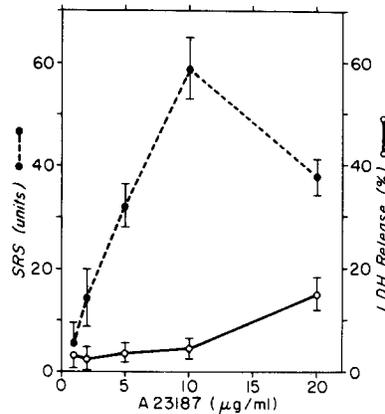


FIG. 1. Effect of ionophore concentration on SRS generation by eosinophils. The reaction mixture contained 5×10^6 eosinophils and the calcium ionophore A23187 at the concentrations indicated in 1.0 ml of the standard salt solution. SRS (●—●) and LDH (○—○) release above background is shown. The background SRS release was 0.2 ± 0.1 units, and LDH release was $2.1 \pm 0.8\%$. The results are the mean \pm SE of five experiments.

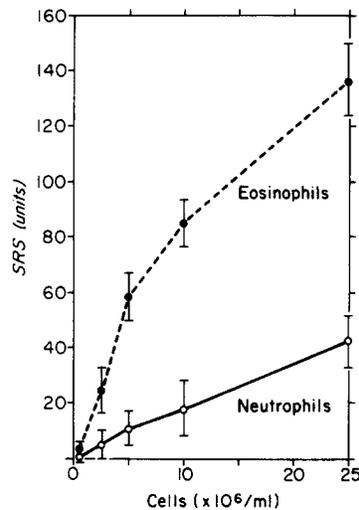


FIG. 2. Comparison of eosinophil and neutrophil SRS generation. The reaction mixture contained eosinophils (●—●) or neutrophils (○—○) in the numbers indicated, $10 \mu\text{g}$ A23187, and the standard salt solution to a final volume of 1.0 ml. Incubation was for 15 min at 37°C . LDH release was $<5\%$ at all cell concentrations and with both cell types. The results are the mean \pm SE of five experiments.

Because SRS is also a product of neutrophil stimulation by A23187, the contribution of the 1–2% neutrophil contamination of the eosinophil preparations was assessed. Fig. 2 compares SRS release by horse neutrophils (98% pure) to that of the eosinophil preparation over a range of cell concentrations from 2.5×10^6 to 25×10^6 per ml. SRS generation by the eosinophil preparations was four to five times that of the neutrophil preparations at all cell concentrations, suggesting that neutrophils do not contribute significantly to the SRS generated by the eosinophil preparations.

The effect of arachidonic acid or inhibitors of arachidonic acid metabolism on SRS

production by eosinophils is shown in Table II. Arachidonic acid at 3.0 μg per ml increased A23187-induced SRS release 31.5%, whereas lower (0.3 $\mu\text{g}/\text{ml}$) or higher (30.0 $\mu\text{g}/\text{ml}$) concentrations had no significant effect. ETYA, an inhibitor of both the cyclooxygenase and lipoxygenase pathways of arachidonic acid metabolism (32), significantly inhibited A23187-induced eosinophil SRS production at concentrations of 10 and 100 μg per ml, whereas indomethacin, a selective inhibitor of the cyclooxygenase pathway (32), increased SRS production at a concentration of 1.0 μg per ml.

In each of eight experiments, 3×10^8 eosinophils were suspended in 30 ml of the standard salt solution and stimulated with 10 $\mu\text{g}/\text{ml}$ A23187. After ethanol extraction and Amberlite XAD-7 chromatography, the components of the preparation were separated by HPLC. Fig. 3 demonstrates a typical elution pattern of the 280 nm absorbing material. Five 280-nm absorbing peaks with elution times between 11 and 26 min (designated I to V) were found to have SRS activity. All compounds were radiolabeled when stimulation of eosinophils by A23187 was performed in the presence of [^{14}C]arachidonic acid. The SRS ileal contracting activity of compounds I, II, and III were not blocked by FPL 55712, whereas 11 ng/ml FPL 55712 produced a 50% reduction of contractions induced by compounds IV and V. Other characteristics of compounds I to V are indicated below.

Compound I. Compound I, which accounted for 1.8% of the total eosinophil SRS activity, had a retention time of 11.9 min under our standard HPLC conditions (Table III). Its UV spectrum was characterized by a peak at 269 nm and two smaller peaks at 259 and 280 ± 1 nm (Fig. 4A). The methyl ester, trimethylsilylether derivative had an equivalent carbon number of 25.0 by gas chromatography and diagnostic ions at M/A 479 (M-15), 404 (M-90), 383, 293, 217, 203, and 129. Compound I co-eluted with 5-(S), 12-(R), 6-*trans*-LTB₄ under each of three HPLC conditions.

Compound II. Compound II accounted for 2% of the total eosinophil SRS activity

TABLE II
Effect of Arachidonic Acid and Arachidonic Acid Inhibitors on SRS Release by Eosinophils

Supplements	SRS units
None	55.8 \pm 3.8
Arachidonic acid (30.0 $\mu\text{g}/\text{ml}$)	53.0 \pm 5.1
Arachidonic acid (3.0 $\mu\text{g}/\text{ml}$)	69.7 \pm 3.5 <0.05
Arachidonic acid (0.3 $\mu\text{g}/\text{ml}$)	58.1 \pm 3.8
ETYA (100.0 $\mu\text{g}/\text{ml}$)	2.7 \pm 1.2 <0.001
ETYA (10.0 $\mu\text{g}/\text{ml}$)	32.3 \pm 5.4 <0.01
ETYA (1.0 $\mu\text{g}/\text{ml}$)	49.1 \pm 2.8
ETYA (0.1 $\mu\text{g}/\text{ml}$)	56.7 \pm 7.2
Indomethacin (10.0 $\mu\text{g}/\text{ml}$)	58.0 \pm 2.9
Indomethacin (1.0 $\mu\text{g}/\text{ml}$)	71.8 \pm 3.1 <0.02
Indomethacin (0.1 $\mu\text{g}/\text{ml}$)	65.4 \pm 3.6

Eosinophils (5×10^6) in 1.0 ml of the standard salt solution were preincubated with the supplements at the concentrations indicated for 5 min at 37°C, after which 10 $\mu\text{g}/\text{ml}$ of A23187 was added. SRS release was determined after an additional 15 min of incubation. Probability values for the difference from eosinophils plus A23187 alone (none) are shown where significant. The results are the mean \pm SE of five experiments.

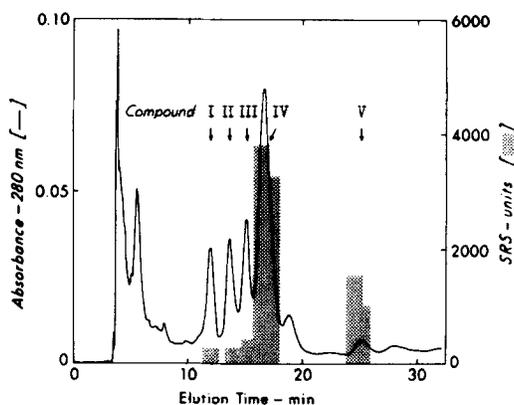


FIG. 3. Reverse-phase HPLC chromatogram of eosinophil SRS. Elution pattern of eosinophil SRS using a μ Bondapak C_{18} column with methanol/water (65:35, vol/vol) and 0.1% acetic acid, pH 3.9, as the solvent. Absorbance was determined at 280 nm, and each 1-ml fraction was assayed for SRS activity.

TABLE III
HPLC Retention Times and SRS Activity of Rechromatographed Eosinophil Compounds

Compound	Retention time			SRS activity
	A	B	C	
		<i>min</i>		<i>% of total</i>
I	11.9	16.8	15.3	1.8
II	13.4	19.2	16.7	2.0
III	14.8	22.2	18.0	3.4
IV	16.4	33.1	6.7	66.0
V	24.9	42.4	15.8	26.8

The retention times of the rechromatographed eosinophil compounds were compared in three different HPLC systems: Column A: 3.9×300 -mm, μ Bondapak C_{18} (10 μ m particles); solvent, methanol/water (65:35, vol/vol) with 0.1% acetic acid, pH 3.9; flow rate, 1.0 ml/min. Column B: 4.6×250 -mm Ultrasphere ODS C_{18} (5 μ m particles); solvent, methanol/water (72:28, vol/vol) with 0.1% acetic acid, pH 3.9; flow rate, 1.0 ml/min. Column C: 4.6×250 -mm, Nucleosil C_{18} (5 μ m particles); solvent, methanol/water (65:35, vol/vol) with 0.01% acetic acid, pH 5.7; flow rate, 1.5 ml/min. The results are the mean of eight experiments.

and had a retention time of 13.4 min under our standard HPLC conditions (Table III). It had the same UV spectrum (Fig. 4A) and identical gas chromatography-mass spectral characteristics as compound I. Compound II co-eluted on each HPLC column with 5-(*S*), 12-(*S*)-6-*trans*-LTB₄.

Compound III. Compound III accounted for 3.4% of the total eosinophil SRS activity and had a retention time of 14.8 min on our standard HPLC conditions (Table III). Its UV spectrum was characterized by a peak at 270 nm, with two smaller peaks at 260 and 281 ± 1 nm (Fig. 4B). Gas chromatography of the derivative indicated an equivalent carbon number of 23.5, and its mass spectral characteristics were identical to that of authentic LTB₄. Compound III co-eluted with LTB₄ under each of three HPLC conditions.

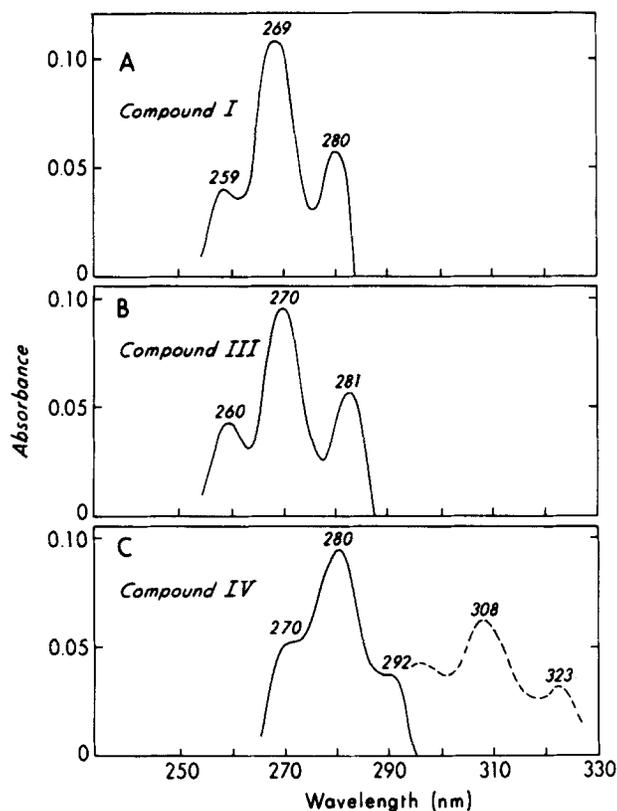


FIG. 4. Ultraviolet spectra of rechromatographed eosinophil SRS fractions. (A), compound I; (B), compound III; (C), compound IV before (—) and after (---) incubation with soybean lipoxygenase. Compounds were dissolved in methanol/water (65:35, vol/vol) with 0.1% acetic acid, pH 3.9.

Compound IV. Compound IV, the predominant eosinophil SRS, as measured by bioactivity (66% of the total SRS), had a retention time of 16.4 min under our standard HPLC conditions (Table III, Fig. 5). Its UV absorption spectrum had a peak at 280 nm, with shoulders at 270 and 292 ± 1 nm (Fig. 4C), which is consistent with a sulfur substituent α to a conjugated triene (33). The absorption spectrum shifted to a peak at 308 nm on incubation of compound IV with soybean lipoxygenase; this enzyme also decreased the ileal contracting activity of compound IV by >90%. Compound IV co-eluted on HPLC with LTC_4 obtained from mouse mastocytoma cells and peritoneal macrophages and also with synthetic LTC_4 (Fig. 5). A small amount of 11-*trans*- LTC_4 was found eluting closely after the eosinophil LTC_4 on rechromatography.

Compound V. Compound V accounted for 26.8% of the eosinophil SRS activity and had a retention time of 24.9 min under our standard HPLC conditions (Table III). It had the same absorption spectrum (Fig. 4C) and response to lipoxygenase as did compound IV. Compound V co-eluted with synthetic and mouse mastocytoma LTD_4 on each of the HPLC columns.

From these findings we conclude that compound I is 5-(*S*), 12-(*R*)-6-*trans*- LTB_4 ; compound II is 5-(*S*), 12-(*S*)-6-*trans*- LTB_4 ; compound III is LTB_4 ; compound IV is

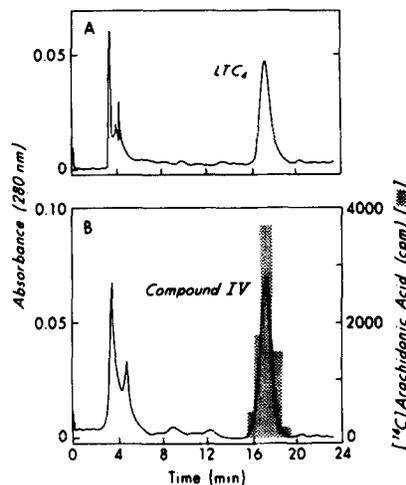


FIG. 5. HPLC identification of compound IV. Rechromatography of (A) synthetic LTC₄ and (B) compound IV on μ Bondapak C₁₈ (10 μ m particles) with methanol/water (65:35, vol/vol) and 0.1% acetic acid, pH 3.9, at a flow rate of 1.0 ml/min. [¹⁴C]arachidonic acid cpm (■) of sequential 0.5-min aliquots of a similarly rechromatographed sample of compound IV are shown.

LTC₄; and compound V is LTD₄. The final yield of each compound from 3×10^8 eosinophils was 0.7 μ g for compound I, 0.8 μ g for compound II, 1.1 μ g for compound III, 3.6 μ g for compound IV, and 0.3 μ g for compound V, as determined from their UV spectra, using the reported extinction coefficients for each of the identified leukotrienes (3, 4, 13, 23). This is an underestimate of the total production because it does not take into account the recovery during the isolation procedures. Beginning with 25,400 SRS units per 3×10^8 eosinophils in the initial supernatant, we recovered 10,500 SRS units in the final HPLC eluates, a recovery of 41%. Horse neutrophils (3×10^8) treated in an identical fashion yielded 0.8 μ g of LTC₄, which corresponds closely to the lower SRS activity produced by neutrophils compared to eosinophils shown in Fig. 2.

Discussion

Our data indicate that horse eosinophils can metabolize arachidonic acid by the lipoxygenase pathway to form leukotrienes with potent SRS activity. Eosinophils highly purified to >98% were found to generate FPL 55712-inhibitable SRS activity without release of appreciable LDH when stimulated with 10 μ g/ml of the calcium ionophore A23187.

Horse eosinophils generated up to five times the amount of SRS produced by similarly treated horse neutrophils when compared on a per cell basis. Thus, of the ~ 138 units of SRS bioactivity generated by 25.0×10^6 eosinophils, <1 SRS unit could be attributed to neutrophil contamination (2% neutrophils each with $\frac{1}{5}$ the activity on a per cell basis). Leukocyte preparations consisting predominantly of neutrophils when incubated with 1–10 μ g/ml ionophore A23187 generate SRS (34) and leukotrienes (3–5). Because these preparations contained a variable proportion of eosinophils, eosinophil contribution to the leukotriene generation may be significant.

The inhibition of eosinophil SRS release by ETYA and augmentation of its release

by arachidonic acid and indomethacin are consistent with other reports (11, 35) that SRS is a lipoxygenase product of arachidonic acid. This was confirmed by separation and identification of specific leukotrienes. Leukotrienes were isolated in pure form by Amberlite XAD-7 and repeated HPLC chromatography. Individual compounds were identified by comparison of their retention times on HPLC under three different conditions to authentic standards, by their characteristic ultraviolet spectra and spectral shift after treatment with soybean lipoxygenase, by their incorporation of [¹⁴C]arachidonic acid, and by gas chromatography and mass spectrometry as indicated in the text. The eosinophil products by these criteria were 5-(*S*), 12-(*R*)-6-*trans*-LTB₄, 5-(*S*), 12-(*S*)-6-*trans*-LTB₄, LTB₄, LTC₄, 11-*trans*-LTC₄, and LTD₄. A23187-stimulated human eosinophils obtained from patients with the hypereosinophilic syndrome produced the same leukotrienes (5-(*S*), 12-(*R*)-6-*trans*-LTB₄, 5-(*S*), 12-(*S*)-6-*trans*-LTB₄, LTB₄, LTC₄, and LTD₄) as did horse eosinophils.² Greater than 92% of the eosinophil SRS bioactivity was contributed by LTC₄ and LTD₄. The myotrophic properties of LTB₄ and its isomers on lung parenchymal strips (7) and guinea pig ileum (36) have only recently been recognized. This property, which was also observed during the course of these investigations, may be due to the release of thromboxanes and prostaglandins induced by LTB₄ in the bioassay system (7, 36).

Thus, our findings suggest that eosinophils, when appropriately stimulated, are a potent source of leukotrienes. Eosinophilia is commonly seen in patients with bronchial asthma (37), where a rise in eosinophil counts has been associated with a deterioration of pulmonary function and a concomitant increase in clinical symptoms (38). Eosinophils may play a causal role in asthma by their capacity to generate leukotrienes with potent SRS activity. Eosinophil production of leukotrienes, which increase vascular permeability and have potent chemotactic and smooth muscle contracting activity, may also be of great significance in the host defense against certain neoplasms and helminths, where eosinophils are found in greatly increased numbers.

Summary

Horse eosinophils purified to >98% generated slow reacting substance (SRS) when incubated with the calcium ionophore A23187. On a per cell basis, eosinophils generated four to five times the SRS produced by similarly treated horse neutrophils. Eosinophil SRS production was inhibited by 5,8,11,14-eicosatetraenoic acid and augmented by indomethacin and arachidonic acid, suggesting that it was a product(s) of the lipoxygenase pathway of arachidonic acid metabolism. Compounds with SRS activity were purified by high-pressure liquid chromatography (HPLC) and identified by ultraviolet spectra, spectral shift on treatment with lipoxygenase, incorporation of [¹⁴C]arachidonic acid, gas chromatography-mass spectrometry, and comparison of retention times on HPLC to authentic standards. The eosinophil products characterized were 5-(*S*), 12-(*R*)-dihydroxy-6-*cis*-8, 10-*trans*-14-*cis*-eicosatetraenoic acid (leukotriene B₄) and its 5-(*S*), 12-(*R*)-6-*trans* and 5-(*S*), 12-(*S*)-6-*trans* isomers, 5-(*S*)-hydroxy-6-(*R*)-*S*-glutathionyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene C₄) and its 11-*trans* isomer, and 5-(*S*)-hydroxy-6-(*R*)-*S*-cysteinylglycine-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (leukotriene D₄).

² Henderson, W. R., J. Harley, A. S. Fauci, and S. J. Klebanoff. Unpublished results.

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