

# ARACHIDONIC ACID METABOLISM BY HUMAN MONOCYTES

## Studies with Platelet-depleted Cultures\*‡

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Murine macrophages synthesize specific cyclo-oxygenase and lipoxygenase metabolites of arachidonic acid (20:4)<sup>1</sup> after stimulation with zymosan (1–3) or particles coated with immune complexes of immunoglobulin G or E (4, 5). In man, less is known about the spectrum of oxygenated 20:4 compounds produced by mononuclear phagocytes. A number of studies have examined the cyclo-oxygenase products of the human monocyte (6–11), the blood-borne precursor of the tissue macrophage. In addition, the synthesis of the lipoxygenase metabolite leukotriene B<sub>4</sub> by the human alveolar macrophage has been reported (12).

A particular problem in the study of 20:4 metabolism by the human monocyte has been platelet contamination. We now report a means of removing platelets adherent to the surfaces of freshly isolated monocytes. As a result, quantitative and qualitative comparisons of the biosynthetic pathways of platelets and monocytes were possible. Thromboxane was the predominant metabolite of monocytes incubated with particulate stimuli whereas exposure to the calcium ionophore A23187 yielded chiefly lipoxygenase products.

### Materials and Methods

*Human Peripheral Blood Components.* Leukocyte concentrates prepared from ACD-A anticoagulated whole blood were obtained from The New York Blood Center, New York, NY. Whole blood was obtained from healthy human volunteers after informed consent and collected into heparin (20 U/ml) or EDTA (final concentration 5 mM).

*Materials.* [5,6,8,9,11,12,14,15-<sup>3</sup>H]20:4 ([<sup>3</sup>H]20:4) (78.2–91.5 Ci/mmol sp act) was

\* Supported by National Institutes of Health grants CA-30198, HL-27186, and AI-07012, and a grant-in-aid from the Squibb Institute for Medical Research.

‡ Presented in part at the Annual Meeting of the American Society for Clinical Investigation, Washington, DC, May, 1982.

§ Recipient of postdoctoral fellowship HD-0578 from the National Institute of Child Health and Human Development (1980–1982).

<sup>1</sup> *Abbreviations used in this paper:* 20:4, arachidonic acid; A23187, calcium ionophore A23187; D-M, detached monocyte culture; [<sup>3</sup>H]20:4, [5,6,8,9,11,12,13,15-<sup>3</sup>H]20:4; HETE, hydroxy-eicosatetraenoic acid; 5-HETE, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5,8,11,13-eicosatetraenoic acid; HPLC, high pressure liquid chromatography; Ig-beads, immunoglobulin G complex-coated Sephadex beads; 6-keto PGF<sub>1α</sub>, 6-keto prostaglandin F<sub>1α</sub>; leukotriene B<sub>4</sub>, 5(S)-12(R)-dihydroxy-6,8,10,14-*cis, trans, trans, cis*-eicosatetraenoic acid; leukotriene C<sub>4</sub>, 5(S)-hydroxy-6(R)-S-γ-glutamylcysteinylglycyl-7,9,11,14-eicosatetraenoic acid; M-P, monocyte-platelet culture; OpZ, opsonized zymosan; PBS, phosphate-buffered saline; PD, calcium- and magnesium-free phosphate-buffered saline; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PGF<sub>2α</sub>, prostaglandin F<sub>2α</sub>; SEM, scanning electron microscopy; SW-M, serum-washed monocyte culture; TLC, thin-layer chromatography; TXB<sub>2</sub>, thromboxane B<sub>2</sub>.

purchased from New England Nuclear, Boston, MA. [5,8,9,11,12,14,15-<sup>3</sup>H] 6-keto prostaglandin F<sub>1α</sub>, [5,6,8,9,11,12,14,15-<sup>3</sup>H] thromboxane B<sub>2</sub>, [5,6,8,9,11,12,14,15-<sup>3</sup>H] prostaglandin F<sub>2α</sub>, and [5,6,8,11,12,14,15-<sup>3</sup>H] prostaglandin E<sub>2</sub> were all purchased from New England Nuclear (all 120–200 Ci/mmol sp act). Hydrofluor was obtained from National Diagnostics, Inc., Somerville, NJ. Ficoll-Paque was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Calcium ionophore A23187 was obtained from Calbiochem-Behring Corp., La Jolla, CA. Serum lipid, Redi-Coat G, and Redi-Coat-2D plates were purchased from Supelco, Inc., Bellefonte, PA. HPLC solvents were either HPLC grade or distilled before use. Zymosan was purchased from ICN K & K Laboratories Inc., Plainview, NY and trypan blue 0.4% in 0.9% NaCl (wt/vol) from Gibco Laboratories, Grand Island, NY. Triton X-100 was obtained from Rohm and Haas Co., Philadelphia, PA. Gas-liquid chromatography columns were purchased from Supelco, Inc., Bellefonte, PA. Heparin (Lipo-Hepin) was obtained from Riker Laboratories, Northridge, CA and Sephadex G-25-40 beads (10–40 μm) were from Sigma Chemical Co., St. Louis, MO.

*Culture Media and Serum.* Adherent cells were cultured in RPMI 1640 supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco Laboratories) and 10–15% human serum. Whole A+ blood was collected without additives (The New York Blood Center) for preparation of the serum used throughout these studies. After 2–4 h at 4°C, clotted blood was centrifuged and the serum supernate filtered (0.45 μM). Aliquots of serum were stored at –20°C before use.

*Preparation of Particles.* Zymosan was prepared as described (1) and was opsonized by incubation at 37°C for 20 min in fresh human serum diluted in normal saline (1:1, vol/vol). The opsonized particles (OpZ) were washed twice and resuspended in RPMI 1640 before use. IgG immune complex-coated Sephadex beads (Ig-beads) were prepared as described previously (4). Sephadex beads containing covalently bound DNP-albumin were the generous gift of Dr. Carol A. Rouzer (The Rockefeller University). Immediately before use, IgG immune complexes were formed on the surfaces of DNP-albumin Sephadex beads by incubation with 100 μg/ml of rabbit anti-DNP IgG at 4°C for 45 min. The beads were washed three times before resuspension in medium.

*Isolation of Adherent Monocytes.* Leukocyte concentrates or anticoagulated whole blood was diluted with cold phosphate-buffered isotonic saline lacking calcium and magnesium (PD) (1:1, vol/vol) and centrifuged at 125–150 g for 20 min at 4°C. All subsequent procedures were carried out at 4°C unless otherwise specified. The supernate was discarded and the cell pellet was washed again in PD. The cells were resuspended in PD containing 0.3 mM EDTA and layered on top of Ficoll-Paque (1.5:1, cell suspension/Ficoll-Paque, vol/vol) before centrifugation at 800 g for 25 min at 23°C (13). The aliquot containing the mononuclear cells at the gradient interface was diluted with PD and pelleted. Two further washes using PD were performed. Mononuclear cells thus isolated were used for establishment of adherent monocyte cultures designated monocyte-platelet (M-P) or processed further to remove monocyte-associated platelets.

To remove platelets specifically adherent to monocytes, gradient-isolated mononuclear cells were resuspended in serum containing 5 mM EDTA (0.7 ml of 77 mM EDTA added per 10 ml of serum) and subjected to two sequential incubations (10–15 min) at 37°C. 10 ml of serum was used per 1–2 × 10<sup>8</sup> mononuclear cells in 15-ml polyethylene conical tubes for each incubation. The cell pellets and mononuclear cells were thoroughly resuspended at the start and end of each incubation. Mononuclear cells were recovered by centrifugation at 400 g for 15 min (23°C). The purified mononuclear cells were resuspended in PD and washed twice at 4°C before plating. Cell counts, cell viability, and cytocentrifugation were performed before and after the serum treatment step. Cell viabilities were routinely ≥98% and total cell and monocyte recoveries were each 90% or greater for this procedure.

For the final isolation of monocytes by adherence, mononuclear cell suspensions washed with PD alone (M-P) or additionally washed in serum-EDTA (serum-washed monocytes, SW-M) were resuspended in medium containing 15% serum (8–10 × 10<sup>6</sup> mononuclear cells/ml) and incubated in sterile 35 mm (1 ml) or 60 mm diam (3 ml) plastic culture dishes for 1–2 h. All incubations were in an atmosphere of 5% CO<sub>2</sub>/95% air at 37°C. Non-

adherent cells were removed by three washes with warm phosphate-buffered saline containing calcium and magnesium (PBS) before incubation with radiolabel or morphologic examination.

*Detachment of SW-M Monolayers.* SW-M could be detached from plastic culture dishes immediately after the initial adherence period. Before detachment, monolayers were washed thoroughly with PBS to remove nonadherent cells. Monocytes were detached by incubation in PD (37°C) for several minutes and gentle repipetting using plastic labware. Viabilities of detached monocytes were >90%. Monocytes readily readhered in medium containing serum (5%). The resulting monolayers (detached monocyte, D-M) showed the same functional characteristics (ingestion of zymosan) as SW-M. Adherent monocytes in platelet-contaminated cultures (M-P) do not detach when incubated in PD under the same conditions.

*Microscopic Examination of Monocyte Cultures.* Cultures of adherent monocytes were established on acid-washed glass coverslips as described above for M-P and SW-M cultures. After removal of nonadherent cells by PBS washes, coverslips were further incubated in medium with 10% serum for 4 h before examinations by microscopy. >99% of adherent cells (SW-M and D-M) thus obtained showed typical morphology of monocytes upon Wright's staining as well as positive reactions for esterase (14) and peroxidase (15). In addition, >99% of the adherent cells were judged viable after incubation with trypan blue and 95% of individual cells ingested two or more opsonized zymosan particles within 60 min.

For scanning electron microscopy, cells on coverslips were fixed in 1.25% glutaraldehyde in 0.1 M sodium cacodylate, washed, dehydrated in ethanol, transferred to amyl acetate, and critical point dried (Hitachi CTI, Tokyo, Japan) in CO<sub>2</sub>. Specimens were coated with gold (Polaron EM coating unit, E5000; Polaron Instruments Inc., Doylestown, PA) and examined with a high resolution Hitachi scanning electron microscope (HHS/2R) at 20 kV, at a tilt angle of 30° (16).

*Adherent Platelet Cultures.* Whole heparinized blood was centrifuged at 150 g for 15 min at 23°C. The platelet-rich plasma was removed and diluted to 10–20% with medium, warmed to 37°C, and thoroughly resuspended. Aliquots of the diluted platelet-rich plasma were incubated on 35 mm (1 ml) or 60 mm diam (3 ml) plastic culture dishes for 1 to 2 h. Adherent monolayers were washed three times with PD before incubation with radiolabel. Adherent monolayers were nearly confluent and well-spread. A small percentage of the initially adherent platelets became rounded and loosely adherent over time but were effectively washed away before stimulation.

*20:4 Metabolism.* Immediately after the 2-h adherence period, the washed monocyte and platelet cultures were incubated for 4 h in medium containing 10% serum and 0.5  $\mu$ Ci/ml of [<sup>3</sup>H]20:4. Aliquots of medium were taken before and after incubation with cells and the percent uptake of radiolabel was calculated from the decrease in radioactivity of the culture medium as determined by liquid scintillation counting. At the end of the labeling period, cultures were washed three times with PBS and overlaid with medium without serum. Calcium ionophore A23187 was added directly to cultures as an ethanol solution (final ethanol concentration, 0.5%). Ethanol added alone was not found to alter cell morphology, viability, or the spontaneous release of incorporated [<sup>3</sup>H]20:4. OpZ was added to cultures at 160  $\mu$ g/ml and Ig-beads suspended in medium were added to cultures in quantities sufficient to entirely cover the adherent cell monolayer.

After the specified incubation periods, medium was removed from culture dishes, placed on ice, and maintained under a nitrogen atmosphere. Aliquots of media were taken for determination of radiolabel contents after centrifugation to remove debris. For each experiment, triplicate or quadruplicate control cultures given no stimulus were included. In each, the spontaneous release of radiolabel into medium was determined. After the removal of medium, control cell monolayers were washed three times with PBS and scraped into 0.05% Triton X-100. Aliquots of cell lysates were used for determination of total protein (17) and radioactivity contents. For each experiment, control cultures were used to establish the total protein and total radiolabel contents of replicate culture dishes to which stimuli were added. Release of radiolabel was calculated from the mean

radiolabel content of the medium of stimulated cultures and the total radiolabel content of control cultures.

Medium from stimulated cultures was centrifuged (150 g for 10 min at 8°C) and transferred to separate tubes. 20:4 and its oxygenated metabolites were extracted by a modification of the procedure described by Unger et al. (18). In brief, 1 vol of ethanol was added and the mixture was acidified to pH ~3.0 with formic acid. Extraction was performed twice with 1 vol each of chloroform and the organic phases were pooled. Recoveries of radiolabel from the medium were routinely >85%. The extracts were dried to nil under reduced pressure or a stream of nitrogen. After resuspension with washes of ethanol/water (4:1, vol/vol) and chloroform/methanol (2:1, vol/vol), extracts were dried again to remove traces of formic acid.

*Separation of <sup>3</sup>H-labeled Metabolites by High Pressure Liquid Chromatography (HPLC).* [<sup>3</sup>H]-20:4 metabolites extracted from media samples were separated and identified by HPLC using three systems. All samples were resuspended in 0.4 ml of the appropriate HPLC starting solvent before chromatography. (a) HPLC system 1. A column (4.6 × 250 mm) of 5 μm Ultrasphere ODS (Altex Scientific, Inc., Beckman Instruments, Inc., Berkeley, CA) was eluted at a flow rate of 1 ml/min with 60 ml of methanol/water/acetic acid (75:25:0.01, vol/vol/vol) followed by 40 ml of methanol/acetic acid (100:0.01, vol/vol). In some experiments, the column effluent was monitored for UV absorbance at 240 nm using a Spectroflow Monitor SF-770 (Kratos, Schoeffel Instrument Division, Westwood, NJ). Fractions of 1 ml were collected. The contents of fractions were dried under a stream of air and the radiolabel content of each was determined in Hydrofluor by liquid scintillation counting. Under these conditions, stable cyclo-oxygenase products [6-keto prostaglandin F<sub>1α</sub> (6-keto PGF<sub>1α</sub>), thromboxane B<sub>2</sub> (TxB<sub>2</sub>), prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>), and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)] eluted at 4–10 min (19–21); di-hydroxy-eicosatetraenoic acids (di-HETEs) at 11–15 min (19–21), 12-hydroxy-heptadecatrienoic acid (HHT) at 16–18 min, mono-hydroxy-eicosatetraenoic acids (mono-HETEs) at 25–50 min (19–21), and unreacted 20:4 at 71–78 min.

Mono-HETE standards were prepared as described previously (21). The molar quantities of 12-hydroxy-eicosatetraenoic acid (12-HETE) relative to 5-hydroxy-eicosatetraenoic acid (5-HETE) were determined during HPLC from relative peak absorbances (240 nm) of each compound.

(b) In some experiments, HPLC system 2 was used for the initial separation of [<sup>3</sup>H]20:4 metabolites. Reversed-phase columns identical to those used in system 1 were eluted at 1 ml/min with 60 ml of methanol/water/acetic acid (65:35:0.1, vol/vol/vol, adjusted to pH 5.4 with NH<sub>4</sub>OH), followed by 40 ml of methanol/water/acetic acid (75:25:0.01, vol/vol/vol), and finally 40 ml of methanol/acetic acid (100:0.01, vol/vol). Under these conditions, stable cyclo-oxygenase products (6 keto-PGF<sub>1α</sub>, TxB<sub>2</sub>, PGF<sub>2α</sub>, and PGE<sub>2</sub>) elute at 10–18 min, di-HETEs at 30–55 min, HHT at 56–64 min, mono-HETEs at 85–105 min, and unreacted 20:4 at 110–120 min.

(c) For the identification of individual cyclo-oxygenase products, the contents of appropriate fractions from HPLC system 1 (4–10 min) or HPLC system 2 (4–18 min) were pooled, dried under reduced pressure, and chromatographed using HPLC system 3. A Waters Fatty Acid Analysis column (3.9 mm × 30 cm) (Waters Associates, Woburn, MA) was eluted isocratically at 2 ml/min with 100 ml of acetonitrile/water/benzene/acetic acid (76.7:23.0:0.2:0.1, vol/vol/vol/vol) (22) followed by 40 ml of methanol/acetic acid (100:0.01, vol/vol). Identification of recovered radiolabeled cyclo-oxygenase products was accomplished by comparison of retention times with authentic <sup>3</sup>H-labeled standards. Using this system, consistent separation of 6-keto PGF<sub>1α</sub> (9–12 min), TxB<sub>2</sub> (22–25 min), PGF<sub>2α</sub> (27–30 min), and PGE<sub>2</sub> (35–38 min) was obtained.

*Extraction and Separation of Cellular Lipids.* After the specified periods of incubation with [<sup>3</sup>H]20:4 in medium containing serum, cell monolayers were placed on ice, washed three times with isotonic saline, and extracted at 4°C following the procedure of Bligh and Dyer (23). Lipid extracts were concentrated under a stream of nitrogen. For separation of neutral lipid, phospholipid, and free fatty acids, aliquots of lipid extracts were spotted onto Redicoat G thin layer chromatography (TLC) plates prepared as

described (24) and developed with diethyl ether. Individual phospholipids were separated on Redicoat 2D TLC plates. Carrier lipid (serum lipid or cell extracts of the macrophage-like line J774) was added for the purpose of detection. Chromatograms were developed in the first dimension with chloroform/methanol/ammonium hydroxide (65:35:5, vol/vol/vol) and in the second dimension with chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5, vol/vol/vol/vol/vol) (25). Lipid-containing regions were visualized by a brief exposure of plates to iodine vapors and were scraped into scintillation vials. Radioactivity was determined in Hydrofluor after the addition of water.

Total cell phospholipids and neutral lipids were separated on columns of activated Unisil (0.4 g), and fatty acid methyl esters were prepared from the total phospholipid and neutral lipid fractions by transesterification in methanolic HCl (26). Methyl esters were then analyzed by gas-liquid chromatography on  $\frac{1}{8}$ -in  $\times$  6-ft columns of 10% SP-2330 on 100/200 Chromosorb WA/A at 180°C with a carrier gas flow rate of 30 ml/min.

*Radioimmunoassay of TxB<sub>2</sub>.* Molar quantities of TxB<sub>2</sub> in media extracts of appropriately stimulated cultures were determined with commercial radioimmunoassay reagents (Thromboxane B<sub>2</sub>, [<sup>3</sup>H]-RIA kit; New England Nuclear) (27). Recoveries of TxB<sub>2</sub> in extracted media were >98% as determined with [<sup>3</sup>H]TxB<sub>2</sub> standards.

## Results

### *Removal of Rosetted Platelets*

Phase contrast microscopy revealed that adherent monocytes derived from peripheral blood mononuclear cell suspensions contained substantial numbers of platelets rosetted to their surfaces. Scanning electron microscopy (SEM) confirmed these observations (Fig. 1A and B) and distinguished rosetted platelets from monocyte membrane ruffling. Such platelets appeared swollen and therefore activated by morphologic criteria. The average number of platelets per monocyte ranged from 3 to 5. Some lymphocytes (4–5 per 100 monocytes) were identified and cell clumping was evident (Fig. 1A and B).

When mononuclear cell suspensions were subjected to two sequential incubations in serum containing EDTA at 37°C before plating, subsequent surface-bound contamination was reduced to 1–2 platelets per 200 monocytes (Fig. 1C and D). Virtually no lymphocytes were seen, cell clumping was absent, and spreading of individual cells was increased.

Adherent monocytes prepared from SW-M, in contrast to monocytes contaminated with rosetted platelets (M-P), were readily detached from plastic surfaces by incubation in Ca<sup>++</sup>- and Mg<sup>++</sup>-free PBS and gentle pipetting. These D-M readhered to plastic surfaces in medium containing serum (5%). After SW-M detachment, a few platelets remained adherent in the original culture dishes. It was likely that these represented platelets that were concealed beneath adherent monocytes.

*Phospholipid Fatty Acid Composition of M-P and SW-M.* Platelets represented 15–40% of the total cell protein in 8 h M-P cultures [3–5 platelets per monocyte, 3.85 ± 1.22 μg protein/10<sup>6</sup> platelets (28, 29), and 45–55 μg protein/10<sup>6</sup> purified monocytes]. However, as shown in Table I, the phospholipid fatty acid composition of M-P was not significantly different from that of SW-M. This suggested a close similarity between monocyte and platelet phospholipid fatty acid compositions and is in agreement with prior analyses (30).

*Monocyte Uptake and Incorporation of [<sup>3</sup>H]20:4 into Phospholipid.* Between 60 and 70% of the [<sup>3</sup>H]20:4 (5–8 nM) provided in serum-containing medium was

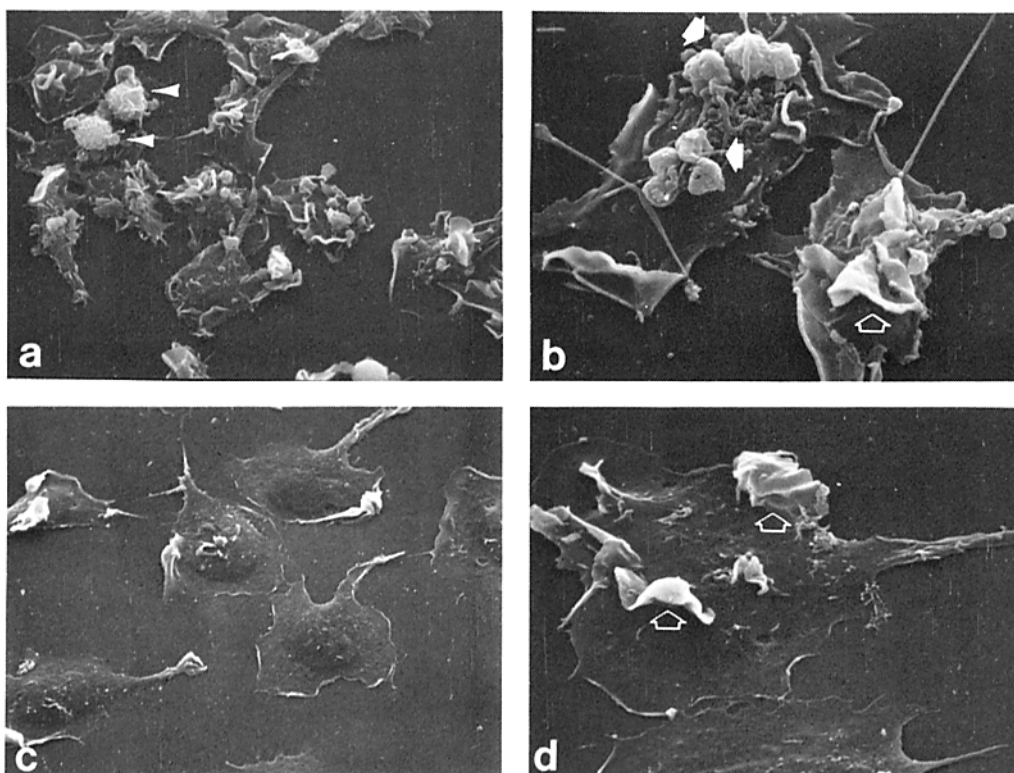


FIGURE 1. SEM of glutaraldehyde-fixed human monocytes. (a) Adherent monocyte culture prepared from peripheral blood mononuclear cells isolated by conventional gradient separation techniques (M-P). Individual monocytes have platelets and occasional lymphocytes (arrows) attached to their surfaces.  $\times 1,000$ . (b) M-P culture containing monocytes with prominent membrane ruffling (open arrow). Variable numbers of rosetted platelets (mean 3–5/monocyte) can be seen (solid arrows).  $\times 3,000$ . (c) Adherent monocyte culture prepared from mononuclear cells washed in serum-EDTA prior to plating (SW-M). The culture contains well-spread monocytes without lymphocyte contamination.  $\times 1,000$ . (d) SW-M culture containing monocytes with prominent membrane ruffling (open arrows), but only 1–2 platelets per 200 monocytes were visible.  $\times 3,500$ .

TABLE I  
*Phospholipid Fatty Acid Compositions of Monocyte Preparations\**

Monocyte preparation	Mole percent of fatty acid				
	16:0	18:0	18:1	18:2	20:4
M-P	24.8 $\pm$ 0.8	21.2 $\pm$ 0.6	20.7 $\pm$ 0.7	7.7 $\pm$ 0.6	25.6 $\pm$ 0.9
SW-M	24.8 $\pm$ 0.8	21.7 $\pm$ 0.4	21.4 $\pm$ 1.1	8.6 $\pm$ 0.6	23.5 $\pm$ 0.9

\*M-P and SW-M cultures were established from a single blood sample as described in Materials and Methods. After an initial 2-h adherence period, cultures were washed and maintained in medium containing 10% serum for an additional 4 h. The adherent cells were then washed thoroughly, placed on ice, and scraped into isotonic saline. Cellular lipids were extracted, and the fatty acid methyl esters were analyzed by gas-liquid chromatography after separation of phospholipids and transesterification. The data represent the mean  $\pm$  SD of seven determinations for each type of monocyte preparation.

TABLE II  
Distribution of Incorporated [ $^3\text{H}$ ]20:4 in Phospholipids of Monocytes  
and Platelets\*

Cell preparation	Percent of total in phospholipids					
	PC <sup>‡</sup>	PE	PI	PS	DPG	SPH
M-P	54.0	20.2	19.7	2.7	2.8	0.6
SW-M	55.4	21.9	18.2	2.7	1.5	0.3
Platelet <sup>§</sup>	49.5	13.2	28.3	5.6	1.4	0.2

\*Cultures were established as described in Materials and Methods. After an initial 2-h adherence period, cultures were washed and incubated with medium containing 10% serum and [ $^3\text{H}$ ]20:4 (0.5  $\mu\text{Ci}$ ) for 4 h. Monolayers were then washed thoroughly and the cellular lipids were extracted after scraping cells into isotonic saline. Aliquots of concentrated extracts were then subjected to two-dimensional thin layer chromatography. The areas of chromatograms containing lipids were scraped and the radioactivity in each was determined. Data are expressed as the percentage of the total radiolabel recovered in all phospholipid species. Results are presented for the mean obtained from two experiments performed in duplicate with M-P and SW-M cultures and one experiment performed in duplicate with adherent platelet cultures. In all determinations for each type of cell preparation, results varied by <4% for each phospholipid species.

<sup>‡</sup>PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidyl glycerol; SPH, sphingomyelin.

<sup>§</sup>The radioactivity recovered in lysophosphatidylcholine and phosphatidic acid represented  $\approx 3\%$  of the total recovered in phospholipids.

taken up in 4 h by M-P and SW-M cultures (45–110  $\mu\text{g}$  protein/dish). Uptake increased progressively to 80% by 12 h with no further increase by 24 h. The absolute amount of radiolabel incorporated on a per cell protein basis was not significantly different in M-P and SW-M cultures containing similar amounts of protein ( $80.8 \pm 11.4$  and  $80.3 \pm 6.9$   $\mu\text{g}$ /dish, respectively).

After a 4-h incubation of M-P or SW-M cultures with [ $^3\text{H}$ ]20:4, 81–85% of the cell-associated radiolabel was esterified into phospholipids. Phosphatidylcholine contained >50% of the label in both M-P and SW-M phospholipids, and the isotope distribution among other phospholipids was similar in SW-M and M-P (Table II). At times up to 24 h, the radiolabel content of phosphatidylethanolamine increased progressively, whereas that of phosphatidylcholine and phosphatidylinositol decreased proportionately.

>95% of [ $^3\text{H}$ ]20:4 taken up by adherent platelets was recovered in phospholipid and its distribution among phospholipid classes was similar to that of adherent monocytes (Table II) and to platelets in suspension (31). This result indicated that platelets contaminating M-P cultures do not alter the distribution of incorporated [ $^3\text{H}$ ]20:4 among cell phospholipids.

*Release of Incorporated [ $^3\text{H}$ ]20:4 by A23187.* Exposure to 1  $\mu\text{g}/\text{ml}$  calcium ionophore A23187 for 20 min released comparable percentages of radiolabel from M-P ( $22.8 \pm 2.8\%$ ), SW-M ( $21.2 \pm 8.1\%$ ), and D-M ( $22.1 \pm 7.5\%$ ) cultures. Release of radiolabel was maximal by 15 min in M-P cultures at ionophore concentrations of 1–10  $\mu\text{g}/\text{ml}$ . Although radiolabel release increased with the dose of A23187 ( $30.3 \pm 2.5\%$  with 10  $\mu\text{g}/\text{ml}$ ), HPLC analyses indicated that

this was mainly accounted for by unreacted 20:4. In comparison, purified platelet monolayers labeled with [ $^3\text{H}$ ]20:4 released  $23.0 \pm 4.4\%$  of their radiolabel content after exposure to A23187 under similar conditions.

It is notable that exposure of monocytes to  $1 \mu\text{g/ml}$  A23187 in serum-less medium reduced cell viability. After 15 min, most monocytes became nonadherent and  $<10\%$  were viable.

*20:4 Metabolism by Monocytes and Platelets Stimulated with A23187.* Fig. 2 compares the HPLC elution profiles of radiolabeled metabolites recovered from the media of M-P, D-M, and platelet cultures treated with A23187. The proportions of cyclo-oxygenase and lipoxygenase products differed for each type of culture (Table III). Platelets converted the greatest percentage of released 20:4 to cyclo-oxygenase products, and 12-HETE represented the single major lipoxygenase product. In monocyte preparations, the proportion of cyclo-oxygenase products decreased by one-fourth with increased monocyte purity (D-M versus M-P). These same cultures also showed a sevenfold reduction in 12-HETE and a 2.3-fold increase in 5-HETE (Table III). As a result, 5-HETE accounted for 75% (SW-M) to 84% (D-M) of the total mono-HETEs in purified monocyte cultures. Measurements of the molar quantities of 5- and 12-HETE from UV absorbance (240 nm) of HPLC effluents confirmed these observations (Fig. 2). The continued production of high levels of cyclo-oxygenase products, di-HETEs, and 5-HETE by SW-M and D-M indicated these were monocyte derived. These data indicated that platelets contribute to the 20:4 metabolites of ionophore-stimulated M-P preparations and that the ratio of 5- to 12-HETE is a convenient monitor of platelet contamination.

*Stimulation with Monocyte- and Platelet-specific Stimuli.* OpZ or Ig-beads promoted the release (15–18%) of [ $^3\text{H}$ ]20:4 from 8-h cultures of M-P, SW-M, and D-M by 60 min. OpZ was readily ingested by monocytes while the large Ig-beads (50  $\mu\text{m}$  diam) remained bound to monocyte surfaces. Incubation of platelets with either particle did not lead to morphologic changes, surface binding, or release

TABLE III  
20:4 Metabolites Released by Monocytes and Platelets Stimulated with A23187\*

20:4 Metabolites	Percent of total oxygenated metabolites			
	Platelets	M-P	SW-M	D-M
Cyclo-oxygenase	$53.9 \pm 4.9$	$31.8 \pm 7.0$	$24.5 \pm 6.0$	$24.2 \pm 8.0$
Mono-HETEs				
12-HETE	$29.7 \pm 8.1$	$20.8 \pm 8.2$	$6.7 \pm 1.8$	$3.2 \pm 0.6$
5-HETE	0	$16.0 \pm 5.3$	$30.0 \pm 3.7$	$36.4 \pm 6.5$
Di-HETEs	$5.0 \pm 1.2$	$21.9 \pm 5.0$	$27.0 \pm 5.0$	$24.4 \pm 8.7$
Other	$11.4 \pm 3.5$	$9.5 \pm 2.2$	$11.8 \pm 3.5$	$11.9 \pm 4.7$

\*Radiolabeled cultures were prepared as described in Materials and Methods. All cultures were stimulated with A23187 ( $1 \mu\text{g/ml}$ ) for 20 min in the absence of serum. Media were extracted and the organic phases were dried and subjected to reverse-phase HPLC using system 1. The radiolabel recovered in individual chromatogram peaks are expressed as percentages of the total oxygenated metabolites recovered. The mean  $\pm$  SD of the results obtained from 5 to 12 determinations for each type of culture are presented. Representative chromatograms are shown in Fig. 2. The elution times of individual 20:4 metabolites in this system are described in the legend of Fig. 1 and Materials and Methods. Cyclo-oxygenase metabolites refer to the total radiolabel content in HPLC fractions in which stable cyclo-oxygenase products (6-keto  $\text{PGF}_{1\alpha}$ ,  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$ ,  $\text{TXB}_2$ , and HHT) elute.



of incorporated [ $^3\text{H}$ ]20:4 above control levels (1–2.5%).

HPLC profiles of radiolabeled compounds produced in response to OpZ (M-P, SW-M, and D-M) and Ig-beads (M-P and SW-M) were similar, but distinct from those from A23187-stimulated monocytes. As shown in Fig. 3, the exposure of M-P or SW-M to OpZ induced the synthesis of mainly cyclo-oxygenase products (47–58% of the total 20:4 metabolites). Substantially smaller amounts of lipoxygenase products (<18% di-HETEs and <12% mono-HETEs) were released than with A23187-challenged cells (Table III).

The adherent platelet response ( $23.6 \pm 6.0\%$  release of cell radiolabel) to thrombin was maximal at 1 U/ml (1–10 U/ml tested) and equivalent to that induced by 1  $\mu\text{g}/\text{ml}$  of A23187 at 20 min ( $23.0 \pm 4.4\%$ ). Identical 20:4 metabolites were released by thrombin- and A23187-treated platelets. No radiolabel release was observed when adenosine diphosphate (5  $\mu\text{M}$ ), collagen (100  $\mu\text{g}/\text{ml}$ ), or epinephrine (5  $\mu\text{M}$ ) was added to the medium of adherent platelets. SW-M responded only slightly to thrombin in keeping with their low platelet levels (Table IV). In contrast, the thrombin response of M-P was >50% of that promoted with the ionophore A23187 (Table IV).

The major radiolabeled metabolites released by thrombin-treated M-P (Fig. 4A) corresponded to those isolated from A23187-stimulated platelets (Fig. 2B). In contrast, the small quantity of radiolabel released by SW-M incubated with thrombin was primarily unreacted 20:4 (Fig. 4A). As has been noted in platelets (32), thrombin treatment inactivated the cyclo-oxygenase and lipoxygenase activities of monocytes. Exposure of M-P or SW-M to A23187 (20 min) immediately after a 20-min thrombin incubation resulted in the release of free [ $^3\text{H}$ ]20:4 (Fig. 4B).

*Cyclo-oxygenase Metabolites Synthesized by Purified Monocytes.* Fig. 5 shows HPLC elution profiles of the cyclo-oxygenase products synthesized by SW-M in response to A23187 and monocyte-specific stimuli (OpZ and Ig-beads). The retention times of individual metabolites were identified by comparison with standards. With each of the three stimuli, similar elution profiles were obtained.  $\text{TXB}_2$  was the major cyclo-oxygenase product (61–81%) irrespective of the applied stimulus.  $\text{PGE}_2$  (10–16%),  $\text{PGF}_{2\alpha}$  (5–13%), and 6-keto  $\text{PGF}_{1\alpha}$  (4–10%) individually accounted for smaller proportions of the total radiolabeled cyclo-oxygenase metabolites. Stimulation with monocyte-specific agents resulted in a higher proportion of  $\text{TXB}_2$  (76–81%) than with A23187 (61%) (Fig. 5). This represented an increase from 3.5 to 8 in the  $\text{TXB}_2/\text{PGE}_2$  ratio. Cyclo-oxygenase and lipoxygenase metabolites synthesized by purified monocytes are summarized in Table V.

The molar quantities of  $\text{TXB}_2$  synthesized by monocytes are shown in Table VI. Removal of platelets from monocytes decreased  $\text{TXB}_2$  synthesis in response to A23187 by 50% (M-P vs. D-M); this was consistent with the radiolabel data (above). However, monocyte thromboxane synthesis was consistently higher with OpZ than with A23187. On a per  $\mu\text{g}$  cell protein basis, the level of  $\text{TXB}_2$  release by monocytes exposed to OpZ was comparable to that of adherent platelets challenged with A23187 or thrombin.

*Effect of Culture Time on [ $^3\text{H}$ ]20:4 Metabolism.* M-P and SW-M cultures were maintained in medium containing serum for various intervals before exposure to A23187. At 8 h in culture, both types of cultures released comparable levels of radiolabel (23–28%). However, with longer times in culture the level of release fell. The loss of response was more rapid in SW-M than in M-P (Fig. 6). Furthermore, the percentage of released radiolabel recovered as unreacted 20:4

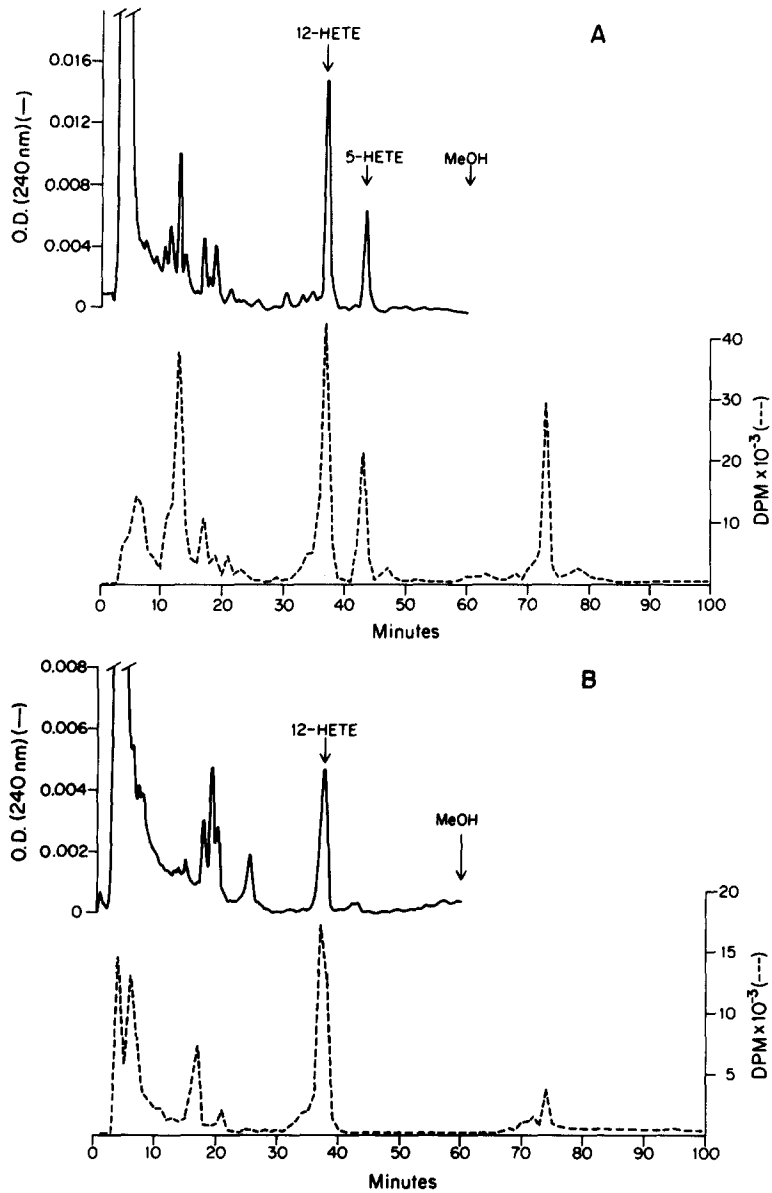


FIGURE 2. Representative HPLC profiles of the 20:4 products released by adherent (A) M-P, (B) platelets, and (C) D-M after incubation with 1  $\mu\text{g}/\text{ml}$  A23187 for 20 min.  $^3\text{H}$  content (---), and UV absorbance (240 nm) (—) of HPLC effluents. Cultures were labeled with [ $^3\text{H}$ ] 20:4, washed, and exposed to A23187 in medium without serum as described in Materials and Methods. After centrifugation, the media were extracted, dried under  $\text{N}_2$ , and subjected to HPLC using system 1. Under these conditions,  $^3\text{H}$ -labeled prostaglandin and thromboxane standards eluted at 4–11 min, di-HETEs at 12–16 min, HHT at 17–18 min, mono-HETEs at 30–50 min, and unreacted 20:4 at 71–80 min. Elution times of 15-HETE (31–33 min), 12-HETE (36–38 min), and 5-HETE (42–45 min) were determined using standards.

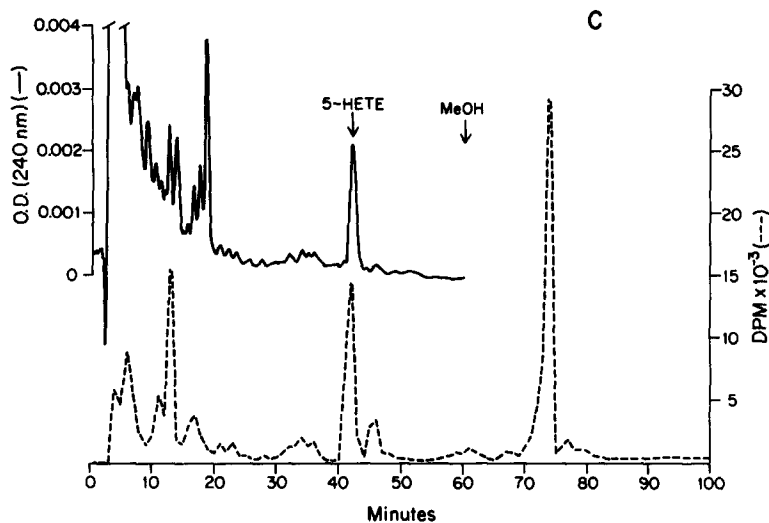


FIGURE 2C

increased as a function of culture time (21% at 8 h, 40% at 24–48 h, and 61% at 120 h in SW-M cultures), indicating that a smaller percentage of the released 20:4 was being metabolized. Culture time also decreased [ $^3\text{H}$ ]20:4 incorporation into the phospholipids of both M-P and SW-M.

It should be noted that culture for 24 h did not result in elimination of platelet contamination in M-P cultures since A23187 stimulation resulted in the same proportion of 12-HETE relative to 5-HETE as in 8-h cultures.

### Discussion

Human monocytes are commonly isolated by density gradient techniques (13) from the mononuclear cell populations of peripheral blood. These contain substantial numbers of free-floating platelets which, because of their buoyant density, can be eliminated by repeated washing in buffer. However, individual adherent monocytes obtained from such preparations have an average of 3–5 platelets specifically rosetted to their surfaces. Rosetted platelets are not removed by simple washing with buffer and represent the major contaminant in the routinely prepared monocytes (M-P) used in this study. These platelets could be dissociated from monocyte surfaces by incubating mononuclear cells in whole serum containing 5 mM EDTA at 37°C. The resulting cultures (SW-M) were shown by SEM to contain <1 platelet per 100 monocytes (Fig. 1C and D). Additional evidence for the absence of platelets was the lack of significant levels of 20:4 release, as opposed to M-P, when exposed to the platelet-specific stimulus thrombin (Table IV).

The basis for platelet adherence to monocyte surfaces and their dissociation in serum-EDTA remains to be determined. However, rosetted platelets consistently showed the morphologic criteria of prior activation. This suggested the involvement of the coagulation process. In this regard, monocytes are obtained in low yields from defibrinated whole blood (13) and both mononuclear phago-

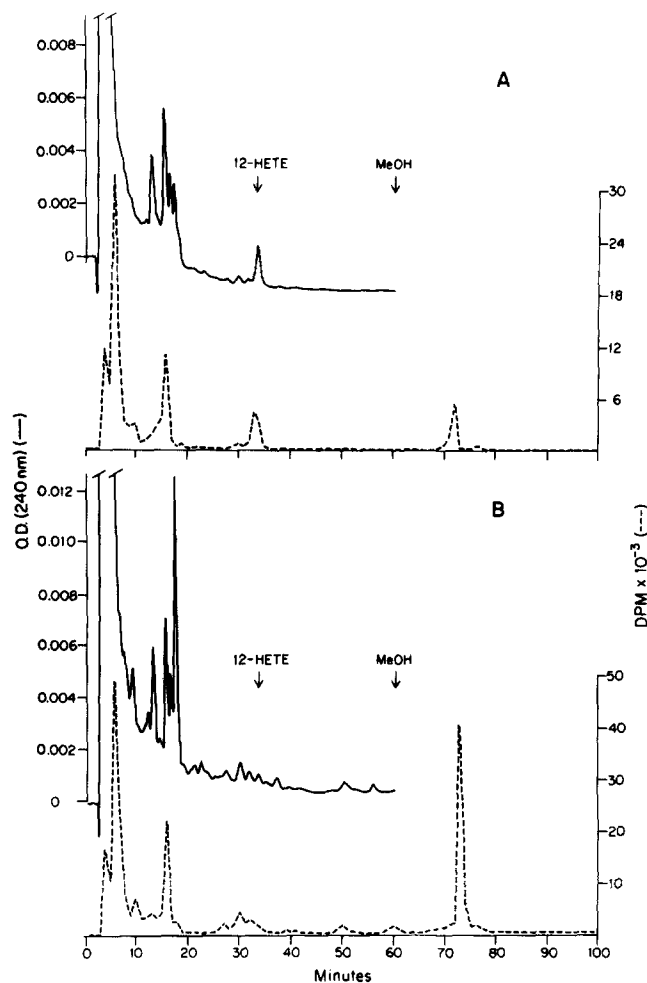


FIGURE 3. HPLC profiles of the 20:4 products released by adherent cultures of (A) M-P or (B) SW-M incubated with OpZ for 60 min. <sup>3</sup>H content (---) and UV absorbance (240 nm) (—) of HPLC effluents. <sup>3</sup>H-labeled cultures were prepared as described in Materials and Methods. Following exposure to OpZ, culture media were centrifuged and extracted. After drying under N<sub>2</sub>, extracts were subjected to HPLC using system 1 as described in Fig. 2. Similar HPLC profiles were obtained with D-M exposed to OpZ and with M-P or SW-M incubated with Ig-beads. Elution times of standards are given in Fig. 2.

cytes and platelets bind fibrin or fibrinogen (33–36).

Our results indicate that platelets can contribute 15–40% of the radiolabeled 20:4 metabolites in short-term mixed cultures (M-P) challenged with A23187 and that platelets remained functional for up to 24 h in culture. Platelets therefore complicate the study of monocyte 20:4 metabolism. The requirement of freshly explanted cells for study emphasized this problem.

The identification of monocyte-specific 20:4 metabolites rested upon the elimination of contaminating platelets. This was especially important since TXB<sub>2</sub>, a well-known product of platelets (37), was the major cyclo-oxygenase product

TABLE IV  
 $[^3\text{H}]20:4$  Uptake and Release by Monocytes\*

Monocyte preparation	Uptake <sup>‡</sup>	Release of incorporated $[^3\text{H}]20:4$ <sup>§</sup>		
		Control	A23187	Thrombin
M-P	9,411 ± 1,095	580 ± 78 (6.2 ± 0.8)	1,795 ± 41 (19.3 ± 0.4)	1,117 ± 65 (12.6 ± 0.7)
SW-M	9,806 ± 2,050	442 ± 31 (4.6 ± 0.4)	1,492 ± 593 (15.4 ± 6.0)	539 ± 33 (5.6 ± 0.4)

\*Each type of culture was prepared as described in Materials and Methods. After a 4-h exposure to  $[^3\text{H}]20:4$  in medium with serum, all cultures were washed and overlaid with medium without serum. Triplicate culture dishes of each cell preparation were incubated for 20 min with medium alone (control), A23187 (1  $\mu\text{g}/\text{ml}$ ), or human thrombin (1 U/ml). The media were then removed, centrifuged to remove cellular debris, and the radioactivity content was determined. Adherent cells from control plates were washed and the radiolabel and protein contents of cell lysates were determined. Results are given for a representative experiment in which M-P and SW-M cultures isolated from a single blood sample contained nearly identical average protein contents per culture dish.

<sup>‡</sup>Uptake (mean ± SD dpm/ $\mu\text{g}$  cell protein) of radiolabel was determined from triplicate control cultures.

<sup>§</sup>Release of incorporated  $[^3\text{H}]20:4$  (mean ± SD dpm/ $\mu\text{g}$  cell protein) was calculated from the radiolabel content of the medium (after incubation with medium alone or the indicated stimulus for 20 min) divided by the mean protein content of the appropriate control culture dishes. The numbers in parentheses are the percent (mean ± SD) of total radiolabel content released.

of monocytes. Once this was achieved, useful biochemical markers of platelet and monocyte metabolism became apparent. As shown previously (37), 12-HETE is the predominant mono-HETE synthesized by platelets, whereas monocytes produced 5-HETE. The ratio of these metabolites then could be employed in monitoring monocyte purification. Low levels of 12-HETE production by SW-M may indicate trace platelet contamination. However, our data do not exclude the possibility that monocytes contain a 12-lipoxygenase.

Platelet- and monocyte-specific stimuli were helpful in identifying the products generated by each cell type. Thrombin promoted the release and metabolism of 20:4 to TXB<sub>2</sub> and 12-HETE by purified adherent platelets. The similar array of oxygenated 20:4 products obtained when M-P were challenged with thrombin were therefore indicative of platelet and not monocyte biosynthesis. The specificity of the thrombin stimulus for platelets was further assured by the lack of response by SW-M and D-M. In contrast, monocytes released high levels of 20:4 metabolites in response to OpZ or Ig-beads, but platelets failed to respond to either particle. Cultures of M-P exposed to these particles generated 20:4 metabolites identical to those of purified monocytes (SW-M and D-M).

Purified monocytes in culture (SW-M and D-M) challenged with particles or A23187 released similar percentages of their radiolabel contents (15–18% vs. 15–25%). After incubation with either OpZ or Ig-beads, 40% of the 20:4 metabolites were cyclo-oxygenase products (Table V). TXB<sub>2</sub> was the single major metabolite (25%) while lipoxygenase products (mono- and di-HETEs) accounted for 30% of the total. In contrast, exposure to A23187 (2  $\mu\text{M}$ ) resulted in the synthesis of predominantly lipoxygenase products (70%, Tables III and V) and cell death. 5-HETE was the single major metabolite (27%) and the proportions of di-HETEs were also high (29%). The human monocyte lipoxygenase therefore

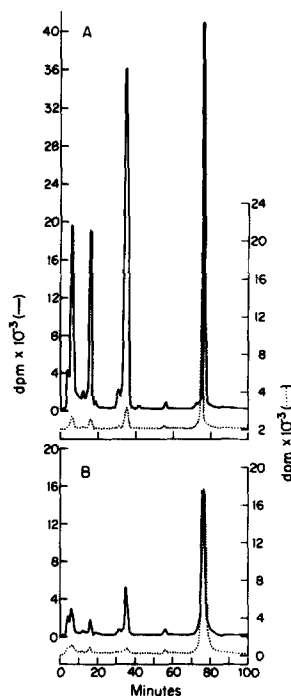


FIGURE 4. HPLC profiles of the [ $^3\text{H}$ ]20:4 products released by (A) M-P and (B) SW-M cultures after sequential treatment with 1 U/ml thrombin for 20 min (—) followed immediately by 1  $\mu\text{g}/\text{ml}$  A23187 (.....).  $^3\text{H}$ -labeled cultures were prepared as described in Materials and Methods. After thorough washing, cultures were overlaid with fresh medium and thrombin was added. At 20 min, the media were removed and replaced with fresh media. A23187 was added immediately and cultures were incubated for an additional 20 min. The media from thrombin and A23187 exposures were centrifuged and extracted. After thorough drying, extracts were subjected to HPLC as described in Fig. 2 (system 1).

has a specificity for the 5 position on the 20:4 molecule. Further evidence in support of this is the production of both leukotrienes  $\text{B}_4$  and  $\text{C}_4$  (to be reported separately). Of the cyclo-oxygenase products generated by A23187-treated monocytes,  $\text{TXB}_2$  was again the predominant metabolite, but it represented a small percentage (7%) of the total 20:4 products. Thus, the balance of 20:4 metabolism via the cyclo-oxygenase and lipoxygenase pathways in the monocyte is influenced by the nature of the stimulus.

Activation of the 20:4 cascade was previously shown in murine macrophages (4) and human neutrophils (38) to require the interaction of ligands with surface receptors. It is of interest that Ig-beads on the cell surface initiated levels of radiolabel release and metabolism equivalent to those of ingestible particles (OpZ), both stimuli leading to the production of a similar array of 20:4 metabolites.

The lipoxygenase products of human monocytes challenged with A23187 are similar to those recovered from human neutrophils incubated with higher concentrations (20  $\mu\text{M}$ ) of the same stimulus and with or without exogenous 20:4 (19). The highest percentage of 20:4 was converted to 5-HETE and a variety of 5, 12 di-HETEs ( $\text{LTB}_4$  and its stereoisomers) (19). However, the spectrum of 20:4

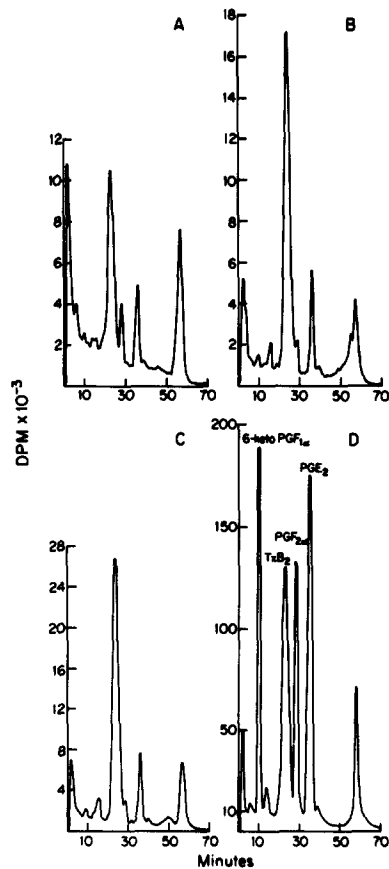


FIGURE 5. Representative HPLC profiles of the <sup>3</sup>H-labeled, stable cyclo-oxygenase metabolites released by cultures of SW-M incubated with (A) 1 µg/ml A23187, (B) OpZ, and (C) Ig-beads. (D) Elution profile of <sup>3</sup>H-labeled standards. Adherent SW-M cultures were established and labeled with [<sup>3</sup>H]20:4 as described in Materials and Methods. After exposure to the indicated stimulus, culture media were centrifuged, extracted, and thoroughly dried before HPLC. The cyclo-oxygenase products initially separated using HPLC system 1 (4–11 min) or system 2 (4–18 min) were dried and rechromatographed using HPLC system 3. Unidentified radiolabeled products elute at the solvent front (2–7 min) and after 50 min.

metabolites released by human neutrophils in response to inflammatory stimuli remain to be determined. Thromboxane is the principal cyclo-oxygenase product of platelets (37) and neutrophils (20, 38) as well as monocytes. Thus, these three classes of circulating cells are strikingly similar in their production of a potent mediator of vasoconstriction and platelet aggregation (39) and have the potential of exerting similar influences within the vasculature during an inflammatory response. From our results and those of others (37, 40), it is evident that the monocyte (0.83 µmol/µg cell protein) compares favorably with the platelet (0.84 µmol/µg cell protein) in its capacity to synthesize TXB<sub>2</sub> (Table VI), whereas the neutrophil (0.003 µmol/µg cell protein) has considerably less synthetic potential (38).

No previous information existed concerning the spectrum of 20:4 metabolites

TABLE V  
 Synthesis of 20:4 Metabolites by SW-M Exposed to A23187 or  
 Particulate Inflammatory Stimuli\*

20:4 metabolites	Percent of total oxygenated metabolites <sup>‡</sup>		
	A23187	OpZ	Ig-Beads
Cyclo-oxygenase			
6-ketoPGF <sub>1α</sub>	1.1	2.0	1.2
TxB <sub>2</sub>	7.1	23.4	24.3
PGF <sub>2α</sub>	1.5	2.6	1.5
PGE <sub>2</sub>	1.9	2.9	3.0
HHT	5.4	16.0	13.0
Total	17.1	46.9	43.0
Lipoxygenase			
mono-HETEs <sup>§</sup>	41.3	≅12.0	≅12.0
di-HETEs	28.9	≅18.0	≅18.0
Total	70.2	≅30.0	≅30.0
Other <sup>¶</sup>	12.7	23.1	26.0

\*Radiolabeled SW-M cultures were established as described in Materials and Methods. After washing, cultures were overlaid with fresh medium and incubated with A23187 (1 μg/ml, 20 min), OpZ (60 min), or Ig beads (60 min). The media were then removed and extracted. The organic phases of extracts were dried and subjected to reverse-phase HPLC with system 2. Retention times of individual products are described in Materials and Methods. Fractions containing cyclo-oxygenase products were rechromatographed using system 3. After HPLC, 75–90% of the radiolabel recovered from medium extracts in all instances represented oxygenated metabolites of [<sup>3</sup>H]20:4.

<sup>‡</sup>Results are presented as the percent of the total oxygenated [<sup>3</sup>H]20:4 products recovered. Of the radiolabel in fractions containing cyclo-oxygenase metabolites, an average of 65–75% represented individual cyclo-oxygenase products identifiable by retention time after rechromatography using HPLC system 3. These data were corrected by this factor. Results are presented for three experiments with A23187 and two experiments with OpZ and Ig-beads. Similar proportions of total cyclo-oxygenase products, mono-HETEs, and di-HETEs were found after HPLC analyses of medium extracts from M-P stimulated with OpZ or Ig-beads (*n* = 2 for each stimulus).

<sup>§</sup>After stimulation of SW-M with A23187, 5- and 12-HETEs each represented an average of 75% and 17%, respectively, of the total mono-HETEs recovered (Table III). In contrast, incubation with OpZ or Ig-beads resulted in substantially smaller amounts of 5-HETE (0.5–5.0%) and 12-HETE (2.5–5.0%) among the total metabolites. The percentages of 5-HETE included that of its δ-lactone form.

<sup>¶</sup>Refers to the sum of the unidentified radiolabeled products recovered in individual chromatograms (system 2). These included very polar material eluting at the solvent front and with prostaglandins (10–16%) and, in addition, presumed mono-HETEs (4–6%) that eluted before 12-HETE and had retention times comparable to 15-HETE. In addition, varying amounts of leukotriene C<sub>4</sub> were recovered and are included in this category.

synthesized by human monocytes. Other studies concerning monocyte 20:4 metabolism have focused largely on the synthesis of PGE<sub>2</sub> (6–11), and through this mediator the monocyte has been implicated in the regulation of immune responses (6) and myelopoiesis (41). Our results show that while the human



TABLE VI  
*Thromboxane Production by Monocytes and Platelets\**

Cell preparation	$\mu\text{mol TXB}_2/\mu\text{g protein}$		
	Thrombin	A23187	OpZ
Platelets	$0.88 \pm 0.18$	$0.80 \pm 0.20$	—
M-P	—	$0.88 \pm 0.46$	$1.49 \pm 0.57$
D-M	—	$0.42 \pm 0.06$	$0.83 \pm 0.28$

\*Adherent cultures of platelets and monocytes were prepared and maintained in culture for 8 h in medium containing serum. Duplicate cultures from each preparation were labeled with [ $^3\text{H}$ ]20:4 and used for concurrent determinations of radiolabel release and products synthesized by HPLC analyses as described in Materials and Methods. After washing, cultures were exposed to the appropriate stimulus (thrombin and A23187 for 20 min and OpZ for 60 min) in medium without serum. The media were then centrifuged and extracted. Prior to radioimmunoassay, extracts were thoroughly dried under  $\text{N}_2$  and resuspended in assay buffer. The results represent the mean  $\pm$  SD  $\mu\text{mol TXB}_2$  produced per  $\mu\text{g}$  cell protein in two (D-M) to four (platelets and M-P) separate experiments. For each monocyte or platelet preparation, assays for  $\text{TXB}_2$  were performed in duplicate with extracts from at least four individual culture dishes given the same stimulus. The results from a single SW-M preparation were similar to D-M.

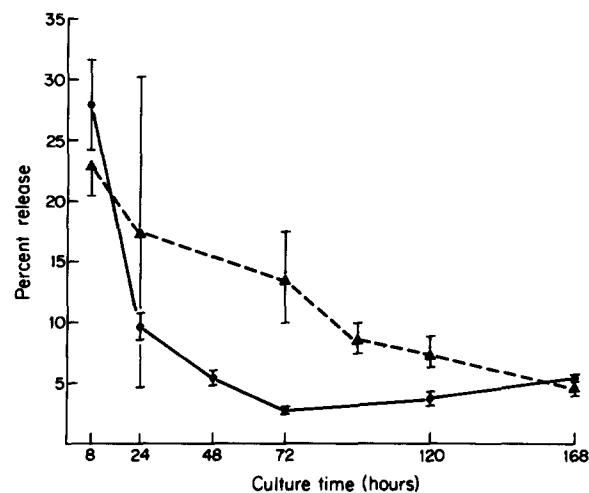


FIGURE 6. Release of incorporated [ $^3\text{H}$ ]20:4 by M-P (—) and SW-M (---) monocyte preparations after increasing intervals of culture. Adherent M-P and SW-M cultures were prepared as described in Materials and Methods. Adherent cells were maintained in medium containing serum (15%) until 4 h before the indicated time points (8, 24, 48, 72, 120, and 168 h). Cultures were then washed and exposed to [ $^3\text{H}$ ]20:4 in medium containing 10% serum for 4 h. Fresh medium was added after thorough washing and cultures were incubated with  $1 \mu\text{g/ml}$  A23187 for 20 min. Radiolabel release was determined by scintillation counting in aliquots taken from centrifuged media. The percentage of radiolabel released was calculated using the total radiolabel content of control cultures given no stimulus.

monocyte produces low levels of PGE<sub>2</sub>, the primary cyclo-oxygenase product formed is thromboxane, regardless of the applied stimulus.

### Summary

Purified human monocytes release and metabolize endogenous arachidonic acid (20:4) from phospholipid stores when challenged with particulate inflammatory stimuli or the calcium ionophore A23187. Using radiolabeled cultures, the percentage of total [<sup>3</sup>H]20:4 released was similar with each type of stimulus. However, the spectrum of 20:4 metabolites differed. With opsonized zymosan (OpZ) or Sephadex beads coated with IgG immune complexes (Ig-beads), the predominant product was thromboxane (25% of the total) together with smaller amounts of other cyclo-oxygenase products and lipoxygenase metabolites. Levels of thromboxane synthesis by monocytes were comparable to those by platelets, as measured by radioimmunoassay. In contrast, exposure to the nonspecific agent A23187 led to mainly lipoxygenase products (70% of the total).

Monocytes isolated from mononuclear cell fractions of peripheral blood contain platelets specifically rosetted to their surfaces. These platelet contaminants were removed by sequential incubations of monocytes in serum and EDTA followed by adherence and detachment from tissue culture vessels. The presence of platelets in routinely isolated monocytes presented a major difficulty in the study of human monocyte 20:4 metabolism since platelets also synthesize thromboxane. Loss of 12-HETE synthesis (16-fold reduction relative to 5-HETE) in A23187-stimulated cultures provided a convenient measure of platelet depletion. This together with the response to monocyte-specific stimuli (OpZ and Ig-beads) allowed for the distinction between monocyte and platelet 20:4 metabolism.

*Received for publication 8 April 1983.*

### References

1. Scott, W. A., J. M. Zrike, A. L. Hamill, J. Kempe, and Z. A. Cohn. 1980. Regulation of arachidonic acid metabolites in macrophages. *J. Exp. Med.* 152:324.
2. Rouzer, C. A., W. A. Scott, A. L. Hamill, and Z. A. Cohn. 1980. Dynamics of leukotriene C production by macrophages. *J. Exp. Med.* 152:1236.
3. Scott, W. A., N. A. Pawlowski, H. W. Murray, M. Andreach, and Z. A. Cohn. 1982. The regulation of arachidonic acid metabolism by macrophage activation. *J. Exp. Med.* 155:1148.
4. Rouzer, C. A., W. A. Scott, J. Kempe, and Z. A. Cohn. 1980. Prostaglandin synthesis by macrophages requires a specific receptor-ligand interaction. *Proc. Natl. Acad. Sci. USA.* 77:4279.
5. Rouzer, C. A., W. A. Scott, A. L. Hamill, F.-T. Liu, D. H. Katz, and Z. A. Cohn. 1982. Secretion of leukotriene C and other arachidonic acid metabolites by macrophages challenged with immunoglobulin E immune complexes. *J. Exp. Med.* 156:1077.
6. Goodwin, J. S., A. D. Bankhurst, and R. P. Messner. 1977. Suppression of human T-cell mitogenesis by prostaglandin. *J. Exp. Med.* 146:1719.
7. Kurland, J. I., and R. Bockman. 1978. Prostaglandin E production by human blood monocytes and mouse peritoneal macrophages. *J. Exp. Med.* 147:952.
8. Morley, J., M. A. Bray, R. W. Jones, D. H. Nugteren, and D. A. van Dorp. 1979. Prostaglandin and thromboxane production by human and guinea-pig macrophages

- and leukocytes. *Prostaglandins*. 17:730.
9. Passwell, J. H., J.-M. Dayer, and E. Merler. 1979. Increased prostaglandin production by human monocytes after membrane receptor activation. *J. Immunol.* 123:115.
  10. Kennedy, M. S., J. D. Stobo, and M. E. Goldyne. 1980. In vitro synthesis of prostaglandins and related lipids by populations of human peripheral blood mononuclear cells. *Prostaglandins*. 20:135.
  11. Bockman, R. S. 1981. Prostaglandin production by human blood monocytes and mouse peritoneal macrophages: synthesis dependent on *in vitro* culture conditions. *Prostaglandins*. 21:9.
  12. Fels, A. O. S., N. A. Pawlowski, E. B. Cramer, T. K. C. King, Z. A. Cohn, and W. A. Scott. 1982. Human alveolar macrophages produce leukotriene B<sub>4</sub>. *Proc. Natl. Acad. Sci. USA*. 79:7866.
  13. Bøyum, A. 1976. Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* 5(Suppl. 5):9.
  14. Li, C. Y., K. W. Lam, and L. T. Yam. 1973. Esterases in human leukocytes. *J. Histochem. Cytochem.* 21:1.
  15. Kaplow, L. S. 1965. Simplified myeloperoxidase stain using benzidine dihydrochloride. *Blood*. 26:215.
  16. Kaplan, G., and K. Berpheussen. 1977. The morphology of echinoid phagocytes and mouse peritoneal macrophages during phagocytosis *in vitro*. *Scand. J. Immunol.* 6:1289.
  17. Lowry, O. H., N. J. Rosenbrough, A. J. Farr, and R. J. Randall. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem.* 193:265.
  18. Unger, W. G., I. F. Stamford, and A. Bennett. 1971. Extraction of prostaglandins from human blood. *Nature (Lond.)*. 233:336.
  19. Borgeat, P., and B. Samuelsson. 1979. Arachidonic acid metabolism in polymorphonuclear leukocytes: effects of ionophore A23187. *Proc. Natl. Acad. Sci. USA*. 76:2148.
  20. Bokoch, G. M., and P. W. Reed. 1980. Stimulation of arachidonic acid metabolism in the polymorphonuclear leukocyte by an N-formylated peptide. Comparison with ionophore A23187. *J. Biol. Chem.* 255:10223.
  21. Pawlowski, N. A., W. A. Scott, M. Andreach, and Z. A. Cohn. 1982. Uptake and metabolism of monohydroxy-eicosatetraenoic acids by macrophages. *J. Exp. Med.* 155:1653.
  22. Alam, I., K. Ohuchi, and L. Levine. 1979. Determination of cyclo-oxygenase products and prostaglandin metabolites using high-pressure liquid chromatography and radioimmunoassay. *Anal. Biochem.* 93:339.
  23. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911.
  24. Keith, A. D., A. S. Waggoner, and O. H. Griffith. 1968. Spin-labeled mitochondrial lipids in *Neurospora crassa*. *Proc. Natl. Acad. Sci. USA*. 61:819.
  25. Rouser, G., S. Fleischer, and A. Yamamoto. 1970. Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorous analysis of spots. *Lipids*. 5:494.
  26. Mahoney, E. M., A. L. Hamill, W. A. Scott, and Z. A. Cohn. 1977. Response of endocytosis to altered fatty acid composition of macrophage phospholipids. *Proc. Natl. Acad. Sci. USA*. 74:4895.
  27. Fitzpatrick, F. A. 1982. A radioimmunoassay for Thromboxane B<sub>2</sub>. *Methods Enzymol.* 86:286.
  28. Nurden, A. T., D. Dupuis, T. J. Kunicki, and J. P. Caen. 1981. Analysis of the glycoprotein and protein composition of Bernard-Soulier platelets by single and two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Clin. Invest.* 67:1431.

29. Marcus, A. J., S. T. Silk, L. B. Safier, and H. L. Ullman. 1977. Superoxide production and reducing activity in human platelets. *J. Clin. Invest.* 59:149.
30. Stossel, T. P., R. J. Mason, and A. L. Smith. 1974. Lipid peroxidation by human blood phagocytes. *J. Clin. Invest.* 54:638.
31. Bills, T. K., J. B. Smith, and M. J. Silver. 1977. Selective release of arachidonic acid from the phospholipids of human platelets in response to thrombin. *J. Clin. Invest.* 60:1.
32. Lapetina, E. G., and P. Cuatrecasas. 1979. Rapid inactivation of cyclo-oxygenase activity after stimulation of intact platelets. *Proc. Natl. Acad. Sci. USA.* 76:121.
33. Colvin, R. B., and H. F. Dvorak. 1975. Fibrinogen/fibrin on the surface of macrophages: detection, distribution, binding requirements, acid possible role in macrophage adherence phenomena. *J. Exp. Med.* 142:1377.
34. Sherman, L. A., and J. Lee. 1977. Specific binding of soluble fibrin to macrophages. *J. Exp. Med.* 145:76.
35. Gonda, S. R., and J. R. Shainoff. 1982. Adsorptive endocytosis of fibrin monomer by macrophages: evidence of a receptor for the amino terminus of the fibrin  $\alpha$  chain. *Proc. Natl. Acad. Sci. USA.* 79:4565.
36. Plow, E. F., and G. Mauguerie. 1982. Inhibition of fibrinogen binding to human platelets by the tetrapeptide glycyl-L-prolyl-L-arginyl-L-proline. *Proc. Natl. Acad. Sci. USA.* 79:3711.
37. Hamberg, M., J. Svensson, and B. Samuelsson. 1974. Prostaglandin endoperoxides. A new concept concerning the mode of action and release of prostaglandins. *Proc. Natl. Acad. Sci. USA.* 71:3824.
38. Goldstein, I. M., C. L. Malmsten, H. Kindahl, H. B. Kaplan, O. Ridmark, B. Samuelsson, and G. Weissmann. 1978. Thromboxane generation by human peripheral blood polymorphonuclear leukocytes. *J. Exp. Med.* 148:787.
39. Hamberg, M., J. Svensson, and B. Samuelsson. 1975. Thromboxanes; a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. USA.* 72:2994.
40. Polley, M. J., R. L. Nachman, and B. B. Weksler. 1981. Human complement in the arachidonic acid transformation pathway in platelets. *J. Exp. Med.* 153:257.
41. Kurland, J. I., L. M. Pelus, P. Ralph, R. S. Bockman, and M. A. S. Moore. 1979. Induction of prostaglandin E synthesis in normal and neoplastic macrophages: role for colony-stimulating factor(s) distinct from effects on myeloid progenitor cell proliferation. *Proc. Natl. Acad. Sci. USA.* 76:2326.