Brief Definitive Report

COMPARTMENTALIZED REGULATION OF MACROPHAGE ARACHIDONIC ACID METABOLISM

BY A. O. S. FELS*‡, N. A. PAWLOWSKI*, E. L. ABRAHAM*, AND Z. A. COHN*

*From the *Department of Cellular Physiology and Immunology, The Rockefeller University, New York 10021; and the ‡Department of Pulmonary Disease, The New York Hospital-Cornell University Medical Center, New York 10021*

Mouse peritoneal macrophages given a phagocytic stimulus release large amounts of cyclooxygenase and lipoxygenase metabolites of arachidonic acid (20:4) (1, 2). After macrophage activation through intraperitoneal administration of inflammatory agents, stimulated 20:4 metabolism by peritoneal macrophages is quantitatively and qualitatively altered (3, 4).

To determine whether downregulation of 20:4 metabolism occurs in macrophage populations outside a local inflammatory site, we have developed a method for injection of particulates into adjacent anatomic compartments containing comparable populations of mononuclear phagocytes. For this purpose we have used the resident macrophage populations that exist in the pleural and peritoneal cavities. Our results indicate that the downregulation of 20:4 metabolism can occur as a localized process involving a contained macrophage population.

Materials and Methods

Anatomic Separation of the Pleural and Peritoneal Cavities. 25–30 gm female ICR mice (Trudeau Institute, Saranac Lake, NY) were given intrapleural (i.pl.) or i.p. injections of 0.2 ml of 2% Evans Blue Dye (Sigma Chemical Co., St. Louis, MO) in isotonic PBS lacking calcium and magnesium (PD). Dissection of the animals revealed local retention of dye in these anatomic compartments.

Culture of Macrophages. Peritoneal cells were harvested as previously described (5). Pleural cells (6) were obtained after harvest of peritoneal cells, after bleeding from the inferior vena cava. Each pleural cavity was lavaged twice with 1 ml of sterile PD. Pleural and peritoneal macrophages were then cultured in α-MEM (Gibco, Grand Island, NY) containing 10% FCS as adherent cells (3).

Activated Macrophage Populations. Activated macrophage populations were obtained at 14 d, unless otherwise noted, after either i.p. or i.pl. injection of mice with 1.4 mg of formalin-killed *Corynebacterium parvum* (Coparvax, Burroughs Wellcome, Co., Research Triangle Park, NC). Resident peritoneal and pleural cells were harvested simultaneously from noninjected mice.

Synthesis of 20:4 Oxygenated Metabolites. Macrophage cultures maintained in α-MEM and 10% FCS were labeled for 16 h with 0.5 μCi of 5,6,8,9,11,12,14,15-[^{3}H]20:4 (82–91 Ci/mmol, sp act; New England Nuclear, Boston, MA). After three washes, the cultures were overlaid with α-MEM (no serum) and 320 μg of zymosan in a 35-mm dish was used as phagocytic stimulus. Media were removed after incubation at 37°C for 90 min. Determination of radiolabel release into the medium and medium extraction were done as previously described (3). Cell protein was determined by the method of Lowry (7).

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Separation of ^{3}H-labeled Metabolites of HPLC. [^{3}H]20:4 metabolites extracted from the media samples were separated and identified using two HPLC systems. In the first system, a column (4.6 × 250 mm) of 5 μm Ultrasphere ODS (Altex Scientific, Inc., Berkeley, CA) was eluted isocratically at a flow rate of 1 ml/min with (*a*) 60 ml of methanol/water/acetic acid (65:35:0.1, vol/vol/vol, adjusted to pH 5.4 with NH_4OH), (*b*) 40 ml of methanol/water/acetic acid (75:25:0.01, vol/vol/vol), and (*c*) 40 ml of methanol/acetic acid (100:0.01, vol/vol). In some experiments the second solvent was omitted. The column effluent was monitored for UV absorbance at 280 nm. The radiolabel content of each fraction was determined. A second HPLC system (8) was used for separation of cyclooxygenase products. Products were identified by comparison of retention times with authentic ^{3}H-labeled standards.

Extraction and Separation of Cellular Lipids. For the analysis of [^{3}H]20:4 incorporated into cellular lipids, extracts of labeled macrophage cultures were subjected to TLC (9). Gas-liquid chromatography (GLC) analysis of fatty acid methyl esters separated from macrophage phospholipids was performed as previously described (9).

Results

In this study we have looked at the macrophage populations of two serous cavities separated by the diaphragm. These populations could be stimulated independently by local injections of *C. parvum.* In each case this lead to a rapid influx of polymorphonuclear leukocytes (PMNs) into the injected, but not the adjacent, noninjected cavity. The number of PMNs peaked at day 7, but was still significantly elevated at day 14. After the injection of the inflammatory agent we compared the 20:4 metabolism in these two macrophage populations.

Phospholipid Fatty Acid Composition and [^{3}H]20:4 Uptake. The phospholipid fatty acid compositions (20:4 was 25 mol-% of the total phospholipid fatty acid content) and phospholipid phosphorus contents (205 ± 10 pmol lipid inorganic phosphate [Pi]/μg cell protein) of the resident pleural and peritoneal macrophages were similar. After labeling of the cells with [^{3}H]20:4 for 16 h, uptake of the radiolabel was >85% and esterification into phospholipid was >85%. Similar uptake and distribution of label into individual phospholipid species was found for inflammatory pleural and peritoneal macrophages.

20:4 Metabolism by Resident Macrophages. After incubation for 90 min with a maximum phagocytic stimulus (zymosan), release of radiolabel by the pleural and peritoneal macrophages was comparable. HPLC analysis of medium extracts showed that >95% of the [^{3}H]20:4 released into the medium by each population was oxygenated metabolites. Both pleural and peritoneal cells released predominantly cyclooxygenase products, 72 and 81% of the total metabolites, respectively (Table I, *a* and *b*). Leukotriene C (LTC) was also a significant metabolite. Interestingly, LTC production by the pleural macrophages was consistantly higher than by peritoneal macrophages (17 ± 5 vs. 12 ± 3% of the total). The calculated (10) molar quantities of LTC secreted by resident pleural and peritoneal macrophages were 3.9 ± 0.13 and 2.8 ± 0.84 pmol/μg cell protein. Integrated UV absorbance measurements of LTC purified by HPLC (Fig. 1), after correction for recoveries (50–60%), were in agreement (>80%) with these calculated values.

Resident peritoneal macrophages synthesize the cyclooxygenase products prostacyclin (recovered as its stable metabolite, 6-keto-$PGF_{1\alpha}$) and PGE_2 in nearly equal proportions (3). Resident pleural cells release similar cyclooxygenase products, as shown in Table I, *a* and *b*. For either resident macrophage type, thromboxane (recovered as the stable metabolite TXB_2) was <3% of the cyclo-

TABLE I

Zymosan-induced 20:4 Metabolism by Macrophages from Injected and Noninjected Cavities vs. Resident Cells

Immunization route and macrophage population	Percent [^{3}H] 20:4 released	Percent of total metabolites released						
		Cyclooxygenase					Lipoxygenase	
		6-keto $PGF_{1\alpha}$	TXB_2	$PGF_{2\alpha}$	PGE_2	HHT	LTC	Mono-Hetes
None								
a PL	24 ± 6	29 ± 5	2 ± 1	2 ± 2	39 ± 5	2 ± 2	17 ± 5	3 ± 2
b PE	21 ± 3	40 ± 6	1 ± 1	2 ± 2	38 ± 4	2 ± 1	12 ± 3	1 ± 1
Intrapleural								
c PL	4 ± 2	8 ± 2	17 ± 5	3 ± 2	49 ± 4	4 ± 1	4 ± 3	8 ± 5
d PE	25 ± 4	41 ± 4	1 ± 1	2 ± 1	33 ± 7	2 ± 2	13 ± 5	2 ± 1
Intraperitoneal								
e PL	10 ± 4	16 ± 3	5 ± 1	3 ± 1	56 ± 3	4 ± 3	5 ± 4	6 ± 1
f PE	5 ± 2	7 ± 2	21 ± 3	1 ± 1	56 ± 8	5 ± 2	4 ± 4	5 ± 3

Macrophages isolated from the pleural (PL) or peritoneal (PE) cavities of animals previously injected with *C. parvum*, as described in Materials and Methods, or noninjected controls were labeled overnight with [^{3}H]20:4, washed, and then challenged with an optimal phagocytic dose of zymosan particles. After 90 min, the percentage of cellular radiolabel content released into medium was determined (3). 20:4 metabolites were extracted from the medium and analyzed by HPLC (3). >98% of the extracted label represented oxygenated metabolites in each case, except for cells isolated from locally injected cavities (*c* and *f*) (83–86%). Individual products are expressed as a percentage of the total oxygenated metabolites. Most of the unidentified products (5–11% of the total) eluted as polar material at the solvent front. All results are expressed as the mean ±SD of four experiments. The radiolabel contents of purified metabolites were used for quantitation (Table II) by the method of Scott et al (3). HHT, 12-hydroxyheptadecatrienoic acid; Mono-Hete, monohydroxyeicosatetraenoic acid.

oxygenase metabolites.

Macrophages from the Inflamed Serous Cavity. As shown in Table I, *c* and *f*, both the pleural macrophages after i.pl. and the peritoneal macrophages after i.p. injections showed a >75% reduction in the amount of [^{3}H]20:4 released in response to maximum phagocytic stimulus. Small increases in the levels of free or unmetabolized 20:4 were found (≤14% of total radiolabel released in each case). LTC synthesis decreased by >75% (pleural) and >67% (peritoneal) (Table I, *c* and *f*). As shown in Fig. 1, the amounts of LTC detectable during HPLC elutions were significantly reduced to <190 pmol/mg cell protein. Both locally activated populations also showed alterations in proportions of cyclooxygenase metabolites, with 6-keto-$PGF_{1\alpha}$ decreased 65–70%, and thromboxane increased 2–5-fold (Table I, *c* and *f*). The molar quantities of thromboxane produced, however, were approximately equivalent to those produced by the resident populations (Table II). These alterations in macrophage 20:4 metabolism were present at 4, 7, and 14 d following the injection of *C. parvum*.

Macrophages Harvested from Noninjected Cavities. Although pleural macrophages from animals injected i.pl. showed maximal downregulation of 20:4 metabolism, no downregulation could be demonstrated in the peritoneal cells harvested from the same mice. Both the release and spectrum of metabolites produced by these cells were comparable to those seen in the resident cells (Tables I *d* and II *d*). As shown in Fig. 1, molar quantities of LTC were at the level of resident cells from uninjected animals. In contrast to this, the pleural macrophages harvested from mice injected i.p. showed partial reduction in 20:4 release (Table I *e*). The percentages of 6-keto-$PGF_{1\alpha}$, PGE_2, and TXB_2

TABLE II
Molar Quantities of Individual Metabolites

Injection	Macrophage population	Amount produced (pmol/mg cell protein)				
		6-keto $PGF_{1\alpha}$	TXB_2	PGE_2	LTC	HHT
None						
a	PL	6,745 ± 1,734	421 ± 236	10,921 ± 703	3,946 ± 1,302	917 ± 131
b	PE	9,327 ± 1,242	260 ± 237	9,436 ± 2,767	2,800 ± 898	376 ± 148
Intrapleural						
c	PL	382 ± 370	158 ± 38	947 ± 507	189 ± 65	232 ± 254
d	PE	11,098 ± 3,348	209 ± 184	10,956 ± 2,601	4,237 ± 678	707 ± 308
Intraperitoneal						
e	PL	1,340 ± 747	487 ± 272	5,447 ± 3,107	260 ± 69	349 ± 307
f	PE	184 ± 116	369 ± 43	1,333 ± 499	157 ± 21	160 ± 152

Purification and quantification of metabolites produced were performed as described in the legend to Table I.

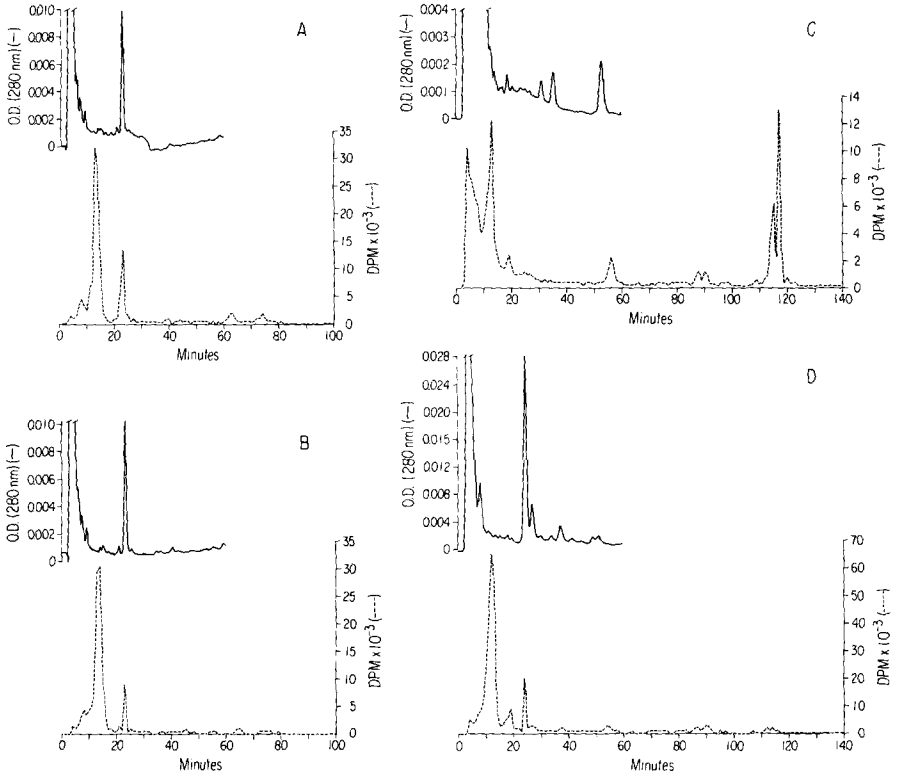

FIGURE 1. HPLC profiles of 20:4 oxygenated metabolites released by resident pleural (*A*) and peritoneal (*B*) macrophages as well as pleural (*C*) and peritoneal (*D*) macrophages from i.pl.-injected mice after a 90-min exposure to 320 μg of zymosan. Media from each set of cultures were pooled, extracted, and chromatographed as described in Materials and Methods. UV absorbance at 280 nm was measured simultaneously. The original macrophage cultures for *A–D* contained 148, 323, 1,329, and 901 μg of protein, respectively. In this system, cyclooxygenase products eluted at 10–18 min, LTC at 20–25 min, and HHT at 56–64 min. Mono-Hetes and unreacted arachidonate eluted at 85–105 min and 110–120 min, respectively, in the 140-min chromatograph, and at 70–80 min and 80–100 in the 100-fraction chromatographs. Synthesis of LTB, -D, or -E was not detected under any of these conditions.

showed a partial shift toward the pattern seen in maximally downregulated cells. (Table I, *a*, *c*, and *e*). However, LTC production by these cells (5%) was similar to that by pleural macrophages after local injection (4%). Individual metabolite production by each macrophage population is summarized in Table II.

Discussion

In this study the availability of two adjacent but anatomically distinct macrophage populations has allowed us to examine whether the downregulation of 20:4 metabolism seen following i.p. challenge with *C. parvum* occurs as a generalized phenomenon or is a localized event. The changes in 20:4 metabolism found in pleural but not peritoneal macrophages harvested from i.pl.-injected animals indicate that downregulation can be a completely localized event not mediated solely by a systemic factor. Although maximal downregulation in pleural cells was seen as early as 4 d after i.pl. injection and persisted for >14 d, the peritoneal macrophages' 20:4 metabolism remained unaffected at all time points.

An intermediate reduction of 20:4 release and a partial modulation of cyclooxygenase products can also occur, as seen in the pleural macrophages following i.p. injection. LTC production in these cells was maximally decreased, suggesting that the lipoxygenase pathway is a more sensitive indicator of this process.

The way in which the i.p. injections affected the partial downregulation of the pleural macrophages is not known. When radioactive *C. parvum* is injected i.p., a small percentage of the radioactivity can be recovered from the lungs (12), suggesting that antigenic spread to the pleural space may have been responsible for the changes in 20:4 metabolism we observed. Labeled *C. parvum* injected i.v. gives an identical distribution of radioactivity (11) but does not cause any alterations in the 20:4 metabolism of pleural cells (our unpublished observations). Alternatively, mononuclear cells sensitized to the antigen may themselves have migrated to the thorax via the bloodstream or the lymphatics.

To date, the factors mediating downregulation have not been identified. Release of cyclooxygenase products by mouse peritoneal macrophages has been shown to correlate closely with the cell's state of activation (3), and it has therefore been proposed that the capacity for 20:4 metabolism is a function of macrophage activation. Crude lymphokine preparations, however, that cause macrophage activation in vitro by gross morphologic, surface antigen, and microbicidal criteria do not cause downregulations (3). Recently IFN-γ, a T cell product, has been shown to be the specific lymphokine responsible for macrophage activation as defined by H_2O_2 production and microbicidal activity (1); however, only modest alterations of 20:4 metabolism are seen in macrophages treated with IFN-γ in vitro (A. Aderem, personal communication). Finally, 20:4 metabolism remains depressed in macrophages long after they have lost the microbicidal capacity of the activated state (3).

Therefore, it is likely that additional inflammatory mediators or cell-cell interactions are involved. It is possible, for example, that PMNs and/or PMN products play a central role in this process, since large numbers of this cell type were present in the injected cavity each time downregulation was observed.

Whatever their cell of origin, the factors that regulate the flux of 20:4 metabolism in macrophages appear to be contained within the local environment.

Summary

We show that downregulation of arachidonic acid (20:4) metabolism which occurs following i.p. injection of *C. parvum* can occur in a single, localized macrophage population, and is therefore unlikely to be mediated solely by a systemic factor.

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Note added in proof: Recent studies (C. S. Tripp, K. M. Leahy, and P. Needleman. 1985. *J. Clin. Invest.* 76:898) of 20:4 metabolism by resident and activated peritoneal macrophages are in agreement with the data in Table II (*b* and *f*) and previous studies (3).

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