BACTERIAL LIPOPOLYSACCHARIDES PRIME MACROPHAGES FOR ENHANCED RELEASE OF ARACHIDONIC ACID METABOLITES

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Macrophages play important roles in inflammation and host defense against bacterial infection. The metabolites of arachidonic acid (20:4)[1] are important modulators of these responses (1). When macrophages interact with stimuli such as an immune complex or a bacterium, phospholipases are activated and 20:4 is liberated from plasma membrane phospholipids. The free 20:4 is then quantitatively oxygenated by either the lipoxygenase pathway to leukotriene C (LTC) and hydroxyeicosatetranoic acids (HETEs) or along the cyclooxygenase pathway to prostaglandin E_2 (PGE_2) and prostacyclin (PGI_2) (2–5).

Very little is known about the cellular events involved in the activation and control of 20:4 metabolism. We report here that bacterial LPS, at concentrations similar to that found in the blood of patients with gram-negative bacteremia (6) prime macrophages for a greatly increased capacity to secrete 20:4 metabolites.

Materials and Methods

Macrophage Cultures. Primary cultures of peritoneal macrophages were established from resident cells of specific pathogen–free female ICR mice (Trudeau Institute, Saranac Lake, NY) weighing 25–30 g as previously described (7). Peritoneal cells (~9×10^6 cells/ml) in α-MEM (Gibco, Grand Island, NY) containing 10% FCS were added to 35-mm-diam plastic culture dishes (1 ml/dish) or to 12-mm glass coverslips (0.1 ml/coverlsip). After 2 h at 37°C in 95% air and 5% CO_2, cultures were washed three times in calcium and magnesium–free phosphate buffered saline (PD) to remove nonadherent cells. Fresh α-MEM plus 10% FCS (1 ml/dish) containing 0.5 μCi of 5,6,8,9,11,12,14,15-[^{3}H]arachidonic acid ([^{3}H]20:4; sp act, 70 Ci/nmol; New England Nuclear, Boston, MA) was added, and the cells were incubated overnight (16 h) (8). Resident peritoneal macrophages were also harvested from Swiss-Webster nude mice (*nu/nu*) (Charles River Laboratory, Wilmington, MA).

LPS Determination. All reagents and culture material were found to contain <0.1 ng/ml contaminating LPS using the limulus amebocyte lysate assay (Cape Cod Associates, Woods Hole, MA).

Priming of Macrophages with LPS. Cells were incubated at 37°C for 60 min (except where indicated) in α-MEM containing *Escherichia coli* 0111:B4 LPS (List Biological Laboratories, Campbell, CA). LPS from *E. coli* 026:B6 (List Laboratories), *S. typhimurium*

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[1] *Abbreviations used in this paper:* 20:4, arachidonic acid; BIg, Ig-coated glass beads; BIgMC, complement-coated beads; HETEs, hydroxyeicosatetranoic acids; LT, leukotriene; PD, calcium and magnesium–free phosphate buffered saline.

(Calbiochem-Behring, La Jolla, CA) and *S. typhosa* (Difco Laboratories, Detroit MI), were also found to prime macrophages for enhanced 20:4 secretion, but most of the experiments reported here used LPS from *E. coli* 0111:B4 (List Laboratories). In experiments where the concentration dependence of LPS was determined, LPS was complexed 1:1 (wt/wt) with delipidated, pyrogen-free human serum albumin (HSA) (Abbot Laboratories, Irving, TX).

Preparation of Unopsonized Zymosan. Zymosan was purchased from ICN (Plainview, NY) and stock solutions in PD were prepared according to the method of Bonney et al. (2).

Preparation of Ligand-coated Particles. Ligand-coated particles were prepared as described previously (9). Briefly, glass beads (8 μm; Duke Scientific Corp., Palo Alto, CA) were derivitized with DNP as described previously for glass coverslips (10), and were suspended in PBS at 50 mg/ml. DNP-beads did not trigger the release of 20:4 from macrophages. IgG-coated beads (BIgG) or IgM-coated beads (BIgM) were obtained by incubating 10 mg of DNP-beads in 250 μl of 10% FCS for 30 min at 20°C with 25 μg/ml of affinty-purified rabbit IgG anti-DNP (11), or with 3 μl of murine monoclonal IgM anti-DNP ascites fluid (12). The beads were then washed three times and suspended at 50 mg/ml in PBS. Complement-coated beads (BIgMC) were prepared by incubating BIgM at 25 μg/ml with 20% normal human serum for 15 min at 37°C followed by three washes in PBS.

BIgMC were shown to interact both C3b and C3bi receptors, since the attachment index of C3b- and C3bi-coated erythrocytes (EC3b, EC3bi) was markedly reduced (~85%) by pretreating the cells with BIgMC (9). BIgM had little effect on the binding of EC3b and EC3bi (9).

Treatment of Macrophages with Latex Particles. Latex beads (1 μm) (Dow Chemical Co., Midland, MI) were washed three times in PD. The particles were centrifuged onto cell cultures at 800 *g* at a concentration of ~50 beads/cell.

"Activation" of Complement Receptors with Lymphokines. To obtain cells that were capable of ingesting C3-coated particles, macrophage monolayers were incubated at 37°C for 16 h in supernatants containing a lymphokine secreted by appropriately triggered T lymphocytes (TCF) (generously provided by Dr. Frank Griffin, University of Alabama, Birmingham, AL) (13). Macrophages thus treated had a phagocytic index of 140 for complement-coated erythrocytes compared, with a phagocytic index of 18 h in untreated cells (9).

Assay of Total [^{3}H]20:4 Release. Macrophages prelabelled with [^{3}H]20:4 (New England Nuclear) were washed four times in PD and were then overlaid with 1 ml of serum-free α-MEM. Zymosan (160 μg/ml), BIgG (5 mg/ml), PMA (100 ng/ml), and A23187 (10 μg/ml) were added as indicated. The cells were incubated at 37°C in 95% air and 5% CO_2 and at the times indicated aliquots of medium were removed and counted in Hydrofluor (National Diagnostics, Inc., Somerville, NJ). The cells were washed in PD and scraped into 1 ml of Triton X-100. Portions of the cell lysates were assayed for radiolabel content, and protein was determined by the method of Lowry et al. (14) with BSA as a standard.

Quantitation of [^{3}H]20:4 Metabolites. The 20:4 metabolites in the culture medium were extracted by the method of Unger et al. (15). To 1 ml of medium was added 1 ml of ethanol and 10 μl of 88% (wt/wt) formic acid, and the resultant solution was extracted twice with 1 ml of chloroform containing 0.005% (wt/vol) butylated hydroxytoluene (Sigma Chemical Co., St. Louis, MO). The lower (chloroform) phases were combined, evaporated to dryness under nitrogen, and the residue was dissolved in the appropriate starting solvent for further purification by reverse-phase HPLC.

Concentrated medium extracts were applied to a column of Ultrasphere C-18 (4.6 mm × 25 cm) (Altex Scientific Inc.) (HPLC system 1). The column was eluted at a rate of 1 ml/min with 60 ml of solvent 1 (methanol/0.01 M H_3PO_4, 65:35, vol/vol, adjusted to pH 5.4 with ammonium hydroxide), followed by 40 ml of solvent 2 (methanol/acetic acid, 100:0.01, vol/vol) (8, 16). For the characterization of the cyclooxygenase metabolites, a duplicate extract was applied on HPLC system 2 using a Waters Fatty Acid Analysis Column (Waters Assoc., Milford, MA). Prostaglandins were eluted at a flow rate of 2

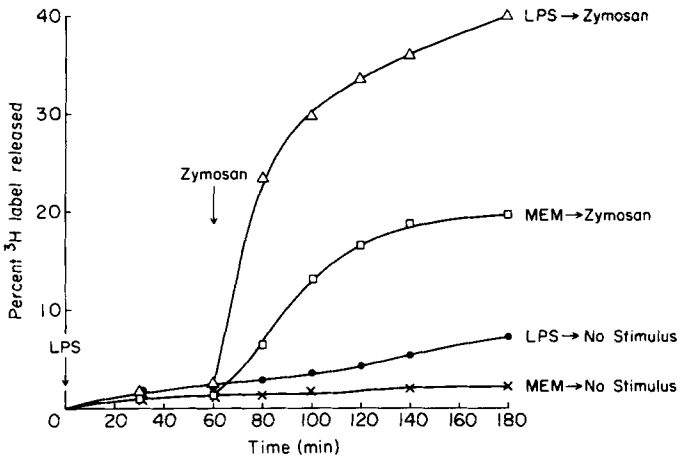

FIGURE 1. Effect of LPS on zymosan-induced 20:4 secretion from resident peritoneal macrophages. Macrophages were isolated and labeled with [^{3}H]20:4. The cells were then washed and overlaid with either α-MEM containing 1 μg/ml LPS (△, ●) or with α-MEM alone (□, ×). After 60 min at 37°C in 95% air/5% CO_2, the cells were treated with 160 μg/ml zymosan as indicated (△, □). At the specified times, duplicate aliquots of the medium were removed and counted. The cells were scraped twice in 0.5% Triton X-100, and the radiolabel and protein content of the cell lysates were determined. Values are expressed as percent of total cellular 20:4 released into the medium, and are means of triplicate cultures.

ml/min with 120 ml of solvent 3 (water/acetonitrile/benzene/acetic acid, 76.7:23:0.2:0.1, vol/vol/vol/vol), followed by 40 ml of solvent 2 (8, 17). Fractions from HPLC were collected at 1-min intervals, and the radiolabel content of whole fractions or aliquots were measured by liquid scintillation counting in Hydrofluor. Corrections were made for counting efficiency (~40%). The recovery of 20:4 metabolites through extraction and purification procedures was monitored using radiolabeled standards (18).

Materials. All materials used were reagent grade. PMA was from Sigma Chemical Co. and A23187 was obtained from Calbiochem-Behring.

Results

Effect of Bacterial LPS on the 20:4 Cascade. Resident peritoneal macrophages cultured in the absence of a stimulus constitutively secrete low levels of 20:4 metabolites into the medium (Fig. 1). When these cells were triggered with zymosan (Fig. 1), PMA (Fig. 2), immune complexes (Fig. 3), or the Ca^{2+} ionophore A23187 (Fig. 4), oxygenated 20:4 metabolites were secreted. *E. coli* LPS (1 μg/ml) was a poor stimulus of 20:4 metabolite release (Fig. 1). After a lag period of 60–80 min, there was a gradual release of ~8% of cellular 20:4, with a maximum at 180 min.

In contrast to the previous results, preincubation of cells with LPS for 60 min had a striking effect on the rate of 20:4 secreted in response to a second stimulus. Thus LPS-primed cells, challenged with zymosan, secreted 23% of their cellular 20:4 by 20 min, compared with 6% released by cells that had not been treated with LPS (Fig. 1). LPS priming also increased the total amount of 20:4 secretion from the cells. Primed cells released 40% of their 20:4 in response to zymosan, while untreated cells were stimulated to secrete a maximum of 20% (Fig. 1).

The priming effect was even more pronounced when the cells were stimulated with PMA (Fig. 2). On its own, PMA triggered the release of ~12% of the

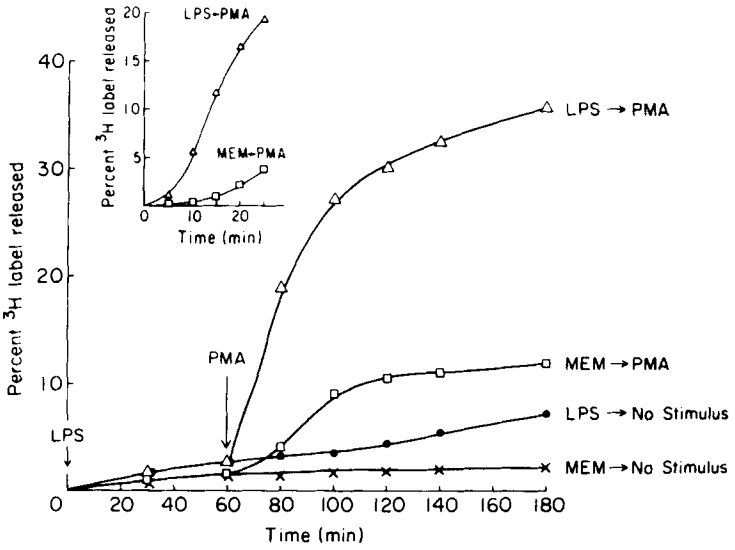

FIGURE 2. Effect of LPS on PMA-induced 20:4 secretion from resident peritoneal macrophages. Macrophages were isolated and labeled with [^{3}H]20:4 as in Fig. 1. Cells were then treated with 100 ng/ml PMA as in Fig. 1 (△, □).

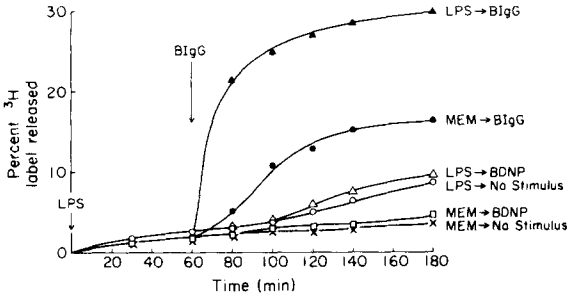

FIGURE 3. Effect of LPS on immune complex-triggered 20:4 secretion from resident peritoneal macrophages. As in Fig. 1, cells were overlaid with either α-MEM containing 1 μg/ml LPS (▲, △, ○) or with α-MEM alone (●, □, ×), then challenged with 5 mg BIgG or with BDNP as indicated. Values were determined and are expressed as described in Fig. 1.

cellular 20:4. However, when first preincubated in LPS for 60 min, 20:4 release in response to PMA was potentiated eight-fold at 20 min, and three-fold at 2 h (Fig. 2). Similarly, LPS also synergized with immune complexes (Fig. 3), and with the Ca^{2+} ionophore A23187 (Fig. 4). As can be noted from Fig. 2 (*inset*) and from Figs. 1–3, 20:4 secretion only commenced after an initial lag phase when unprimed cells were stimulated. This lag phase was much reduced in LPS-treated cells.

These data indicate that, while LPS alone is a poor trigger of the 20:4 cascade, it does prime macrophages for greatly enhanced 20:4 release in response to a variety of particulate and soluble stimuli.

Concentration Dependence and Temporal Response of LPS Effect. The concentration dependence of LPS-induced triggering of 20:4 release and LPS-induced priming of macrophages for enhanced 20:4 secretion with PMA was determined

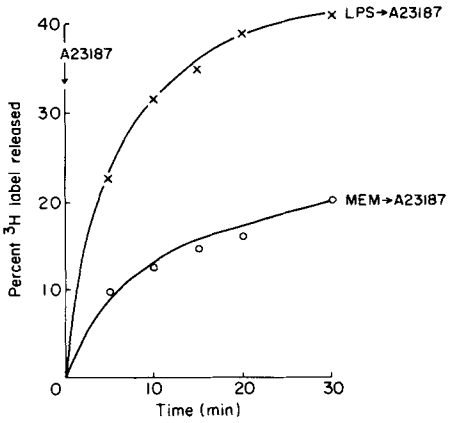

FIGURE 4. Effect of LPS on A23187-induced 20:4 secretion from resident peritoneal macrophages. Washed and preincubated for 60 min in α-MEM containing 1 μg/ml LPS (×) or in α-MEM alone (○), the cells were challenged with A23187) (10 μg/ml), and 20:4 secretion was determined as in Fig. 1.

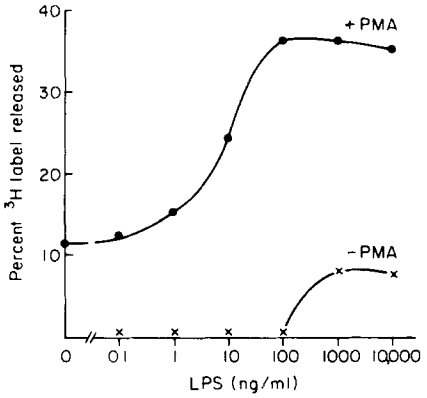

FIGURE 5. Concentration dependence of LPS-induced 20:4 secretion and of LPS-priming of macrophages for enhanced 20:4 secretion in response to PMA. The cells treated with the indicated concentration of HSA-LPS suspended in α-MEM for 180 min, at which point 20:4 release was assessed (×). Alternatively, cells were treated with the concentration of HSA-LPS indicated for 60 min, after which the cells were challenged with 100 ng/ml PMA for a further 120 min (●). The amount of radiolabeled 20:4 released into the medium was determined. Values are expressed as percent of total cellular 20:4 released into the medium, and are means of triplicate cultures.

(Fig. 5). Interestingly, the half-maximal effects of the two parameters were different. LPS-induced 20:4 secretion had a $K_{1/2}$ of 500 ng/ml, while LPS-dependent priming had a $K_{1/2}$ of 10 ng/ml. The half-maximal concentration of LPS required for enhanced 20:4 secretion in response to a second stimulus was the same irrespective of which stimulus was used (Table I).

The time-dependence of LPS priming was also investigated (Fig. 6). Pretreatment of cells for 0–10 min with LPS produced no augmentation of PMA induced 20:4 release. Preincubation of cells for periods longer than 10 min resulted in enhanced PMA triggered 20:4 secretion (Fig. 6). Maximal priming was obtained after 50–60 min preincubation with LPS. The time dependence of LPS priming

TABLE I

Concentration and Time Dependence of LPS Priming of Macrophage for Enhanced 20:4 Metabolism

Stimulus*	$K_{1/2}$‡	$t_{1/2}$§	Priming after 30 h (fold increase)‖
	ng/ml	*min*	
Zymosan	8.5	18	1.7
BIgG	5.1	20	2.3
PMA	10.8	21	3.0
A23187	7.0	22	2.8

Resident peritoneal macrophages were isolated and labeled overnight with [^{3}H]20:4. The cells were then washed, and the concentration and time dependence of LPS priming of the cells for enhanced 20:4 secretion was determined as described in the legends to Figs. 5–7.

* Cells were challenged with zymosan (160 μg/ml), BIgG (5 mg), or PMA (100 ng/ml) for 120 min, or with A23187 (10 μg/ml) for 30 min.

‡ The half-maximal concentration of LPS required for priming.

§ The preincubation time required for half-maximal activation of 20:4 metabolism.

‖ Cells were primed with LPS and washed as described in the legend to Fig. 7. The cells were then challenged with the indicated stimulus after a further 30 h in culture. The data is expressed as the amount of 20:4 released by LPS-primed cells divided by the amount of 20:4 released by cells that had not been exposed to LPS.

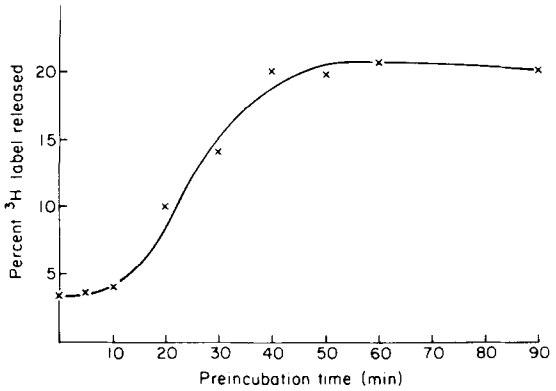

FIGURE 6. 20:4 secretion by macrophages preincubated for varying times in LPS. Cells preincubated with 100 ng/ml HSA-LPS for the times indicated were treated with 100 ng/ml PMA for 15 min, after which duplicate aliquots of the medium were removed, counted and assayed for 20:4 secretion as in Fig. 1.

of the 20:4 cascade was similar regardless of which trigger was used to stimulate 20:4 secretion (Table I).

We also attempted to examine how long the primed state persisted. Cells were treated with LPS for 1 h, then washed with PD containing delipidated BSA, and PMA-triggered 20:4 release was determined (Fig. 7). Enhanced levels of 20:4 release were maintained for at least 30 h. Macrophages remained primed for enhanced 20:4 secretion 30 h after addition of LPS irrespective of whether they were challenged with zymosan, immune complexes, PMA, or A23187 (Table I).

These data indicate that the temporal response and concentration dependence

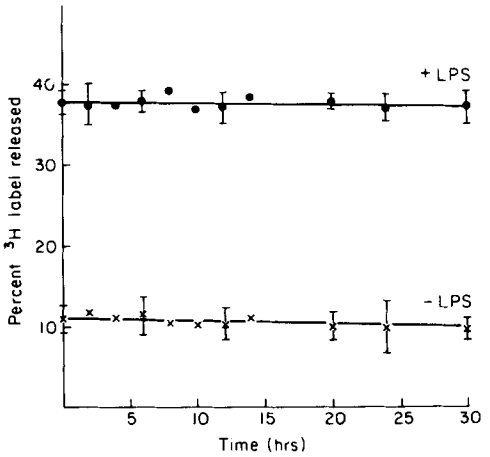

FIGURE 7. Persistence of LPS-priming of macrophages for enhanced 20:4 secretion. The cells were treated with either α-MEM containing 100 ng/ml LPS (●) or with α-MEM alone (×). After 60 min at 37°C in 95% air/5% CO_2 the cells were washed 4 times with PD containing delipidated HSA. The cells were then overlaid with α-MEM containing 10% FCS and [^{3}H]-20:4, and after the specified times, the cells were challenged with 100 ng/ml PMA for 120 min, then assayed as before (Fig. 1).

of LPS priming is identical irrespective of which trigger is used, and suggests that a common mechanism underlies LPS priming for a variety of triggers.

Effect of LPS on Profile of 20:4 Metabolites Secreted by Macrophages. Resident peritoneal macrophages have the capacity of oxygenate 20:4 via the cyclooxygenase pathway (prostacyclin, PGE_2, and HHT) and via the lipoxygenase pathway (LTC and HETEs) (2–5) (Table II). The different trigger stimulated the production of different 20:4 metabolites. Hence zymosan and immune complexes resulted in the secretion of: 6-keto-$PGF_{1\alpha}$, 40%; PGE_2, 26–35%; LTC, 15–25%; HETEs, 10%, and free 20:4, 1% (Table II). On the other hand, PMA and LPS (used as a trigger) only stimulated the release of the cyclooxygenase products 6-keto-$PGF_{1\alpha}$ (55%) and PGE_2 (45%) (Table II). The Ca^{2+} ionophore, A23187, triggered the secretion of proportionately more lipoxygenase products (6-keto-$PGF_{1\alpha}$, 30%; PGE_2, 20%; LTC, 25%, HETEs, 20%, and free 20:4, 5%) (Table II).

Cells primed with LPS for 60 min and then challenged with the stimuli described in Table II secreted the same profile (ratio) of 20:4 metabolites as did unprimed cells, even though larger amounts of each metabolite was secreted by the LPS-treated cells.

Further evidence that LPS treatment did not modulate the cyclooxygenase or lipoxygenase pathways came from experiments investigating the exogenous metabolism of 20:4. Resident peritoneal macrophages metabolize exogenously added 20:4 in the absence of a trigger (19) (Table III). Exposure of macrophages to 0.5 μCi (8.3 nM) [^{3}H]20:4 in serum-free α-MEM for 20 min resulted in the production of 58% cyclooxygenase products (major species 6-keto $PGF_{1\alpha}$), 22% HETEs, and 20% unreacted 20:4. When the experiment was carried out with cells primed with *E. coli* LPS (1 μg/ml), 56% of the label had been metabolized

TABLE II

20:4 Metabolites Synthesized by LPS-primed and Control Macrophages, in Response to a Variety of Stimuli

Condition	Percent ^{3}H label released	6-keto-$PGF_{1\alpha}$	PGE_2	LTC	HETEs	Unreacted 20:4
Zymosan	23.3	40.9	26.0	24.7	7.7	0.7
LPS/Zymosan	42.8	39.5	26.2	23.0	10.1	1.2
BIgG	16.1	40.3	34.9	15.1	8.6	1.1
LPS/BIgG	30.0	42.0	33.9	14.3	9.0	0.8
PMA	10.8	54.0	43.6	<0.2	1.6	0.8
LPS/PMA	37.8	53.7	44.3	<0.2	1.1	0.9
A23187	21.4	30.7	20.5	25.1	18.9	4.8
LPS/A23187	41.0	33.1	21.2	23.2	17.4	5.1
Latex	1.7	57.9	33.7	<0.2	1.9	6.5
LPS/Latex	24.6	54.1	43.5	<0.2	1.2	1.2
LPS alone	8.1	55.3	42.2	<0.2	2.3	0.2

Macrophages were isolated and labeled with [^{3}H]20:4. The cells were washed and preincubated for 60 min with α-MEM containing 1 μg/ml LPS, or with α-MEM alone. The macrophages were then challenged with zymosan (160 μg/ml), BIgG (5 mg), PMA (100 ng/ml), or latex particles for 120 min, or with A23187 (10 μg/ml) for 30 min. The data reported for LPS alone reflects the profile of 20:4 metabolites determined in the medium 180 min after the addition of LPS. After the cells were challenged for the apppropriate times with each stimulus, the medium was aspirated and extracted for 20:4 metabolites. Extracts were dried under N_2 and chromatographed on HPLC systems 1 and 2 as described in Materials and Methods. Values are expressed as the percent of the total [^{3}H]20:4 products formed and calculated on the basis of discernible peaks above background. The data represents the mean ± SD of four determinations.

TABLE III

Effect of LPS on Production of Metabolites from Exogenous 20:4

Condition	Percent [^{3}H]20:4 incorporated by macrophages	Percentage of radiolabel in medium		
		Prostaglandins	HETEs	20:4
Control	39.2 ± 6.1	57.8 ± 8.1	22.0 ± 3.1	20.2 ± 4.0
Preincubated with LPS	43.1 ± 3.2	56.2 ± 6.8	16.5 ± 1.9	27.3 ± 5.1

Macrophages were isolated and incubated overnight in MEM plus 10% FCS. Cultures were washed and preincubated for 60 min with α-MEM containing 1 μg/ml LPS or with α-MEM alone. [^{3}H]20:4 (0.5 μCi, 8.3 nM) was added to the medium, and after 20 min at 37°C the medium was removed and an aliquot was counted. Macrophages were scraped into 1 ml of 0.05% Triton X-100 and the radiolabel content was determined. Data are reported as duplicate determinations on four dishes (mean ± SD) of the total recovered [^{3}H]20:4. The medium was extracted for 20:4 metabolites as described in Materials and Methods. Medium extracts of duplicate 35-mm culture dishes were pooled and subjected to HPLC. The data represent the mean ± SD of four determinations, and are presented as the percent of the total [^{3}H]20:4 recovered in HPLC effluents.

to cyclooxygenase products (mainly 6-keto-$PGF_{1\alpha}$), 16% to HETEs, and 27% was unreacted 20:4 (Table III).

These data suggest that LPS priming promotes the release of greater amounts of 20:4 from membrane phospholipids, but that it does not modulate the 20:4 cascade at the level of either the cycloxygenase or lipoxygenase pathways.

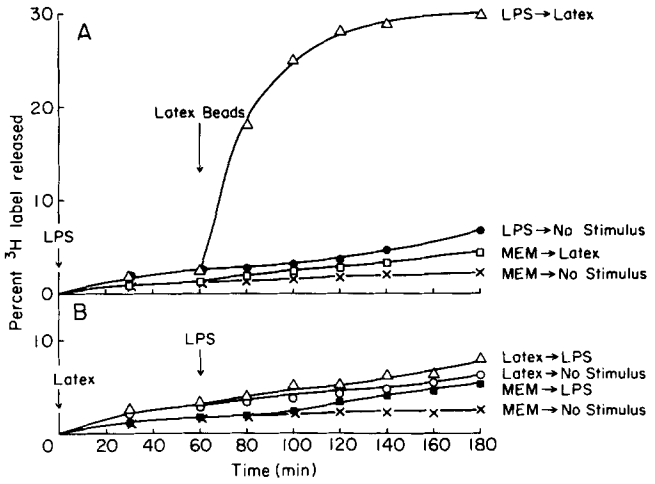

FIGURE 8. Macrophages primed with LPS secrete 20:4 metabolites when challenged with latex beads. Cells were treated and assayed as in Fig. 1. In *B*, the latex particles were added first and LPS was added second.

LPS Primes Macrophages to Secrete 20:4 Metabolites in Response to Latex Beads. Priming with LPS also had the ability to convert a particle without triggering activities into an active one.

Latex particles were ingested by macrophages without the release and metabolism of 20:4 (20), (Fig. 8*A*). However, when LPS-primed cells ingested latex beads, up to 30% of the cellular 20:4 was released (Fig. 8*A*). The metabolites consisted solely of the cyclooxygenated products prostacyclin and PGE_2 (Table II). The order in which the cells were exposed to LPS and latex was critical for the final outcome. When latex beads were added to the cells first, and LPS was added thereafter, no 20:4 metabolites were released (Fig. 8*B*).

These data suggest that stimulus-dependent 20:4 release is composed of two distinct steps, a priming step and a triggering step. LPS can prime the cell for 20:4 secretion, but is inefficient as a trigger. On the other hand, latex can trigger 20:4 secretion, but is incapable of priming the cells for 20:4 secretion.

Complement Receptors Do Not Trigger 20:4 Metabolite Secretion in LPS-primed Macrophages. The complement receptors (both C3b and C3bi) of murine resident peritoneal macrophages have been shown to exist in one of two states. In the first, they are capable of binding particles opsonized with either C3b or C3bi, but are incapable of promoting the phagocytosis of these particles (21). Treatment of the cells with a lymphokine results in their conversion to the second state, capable of both binding and ingesting complement-coated particles (21).

We have shown previously (9) that neither the ligated C3b nor C3bi receptor is capable of triggering 20:4 secretion irrespective of whether these receptors promote phagocytosis. We therefore wished to examine whether priming with LPS permitted these receptors to promote 20:4 secretion.

Particles coated with the complement components C3b and C3bi were incapable of triggering 20:4 metabolite secretion in either LPS-primed or untreated macrophages (data not shown). We next investigated the release of 20:4 from macrophages treated with the lymphokine (13, 21), which renders the cells

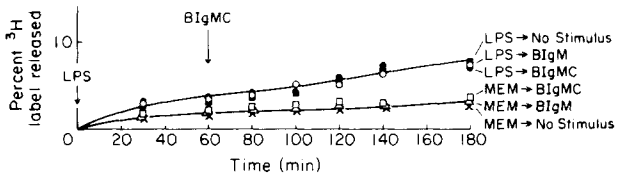

FIGURE 9. Macrophages primed with LPS do not secrete 20:4 metabolites when their complement receptors are ligated. Macrophages were isolated, treated with lymphokine to activate the complement receptors as described in Materials and Methods, and labeled with [^{3}H]20:4. The cells were then washed and treated with either α-MEM containing 1 μg/ml LPS (○, ●, ■) or with α-MEM alone (□, △, ×). After 60 min at 37°C in 95% air/5% CO_2 the cells were challenged with 5 mg of BIgM (△, ■) or BIgMC (●, □). Control cells received no further stimulus (×, ○). Assay procedures were as described in Fig. 1.

capable of promoting phagocytosis via their complement receptors. Ligation of C3b and C3bi receptors in such lymphokine-treated macrophages again failed to trigger the 20:4 cascade in either LPS-primed or unprimed cells (Fig. 9). These data show that release of 20:4 is not the inevitable consequence of phagocytosis or receptor ligation in LPS-primed macrophages. Rather, some particles that do not usually induce 20:4 release (e.g., latex beads) can do so when cells are primed with LPS, but other particles (e.g., C3-coated beads) cannot induce 20:4 release in the absence or presence of LPS.

The Role of T Cells in LPS Priming of 20:4 Metabolism in Macrophages. We wished to investigate whether LPS was acting directly on the macrophages, or whether it was promoting the release of lymphokines from adherent T cells that in turn primed the macrophages.

The protocol for labeling resident peritoneal macrophages with [^{3}H]20:4 necesitates their culture for at least 24 h. Adherent cell populations maintained in culture for 24 h contain very few contaminating T lymphocytes (<5%, data not shown). We excluded the possibility that these contaminating T cells had a role in LPS-induced priming of 20:4 metabolism in macrophages in two ways. First, we showed that the addition of freshly isolated nonadherent cells to macrophage cultures had no effect on LPS priming (data not shown). Second, we demonstrated that peritoneal macrophages isolated from nude mice (Swiss-Webster *nu/nu*), which lack mature T lymphocytes, respond normally to LPS (Fig. 10).

Mature T lymphocytes therefore do not appear to have a role in LPS priming of macrophages for enhanced 20:4 secretion.

Discussion

Murine resident peritoneal macrophages constitutively secrete low levels of 20:4 metabolites including PGI_2, PGE_2, and LTC (2–5). When these cells interact with immune complexes, zymosan, PMA, or the Ca^{2+} ionophore A23187, phospholipases are activated and the cells release large quantities of 20:4 metabolites. LPS has also been shown (22) to trigger the secretion of smaller quantities of 20:4 metabolites consisting exclusively of prostaglandins. We now report that, while LPS alone is a very poor trigger of the 20:4 cascade, it does prime macrophages for greatly enhanced 20:4 metabolite secretion when these cells are challenged with a second stimulus. Thus preincubation of macrophages for

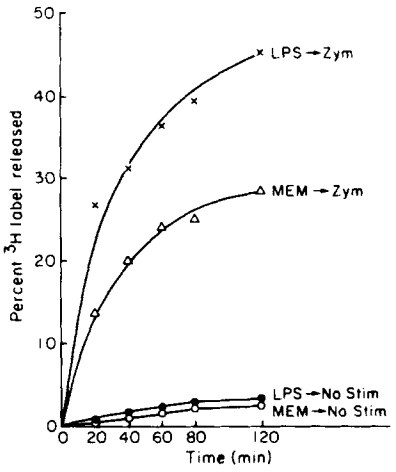

FIGURE 10. Effect of LPS on zymosan-induced 20:4 secretion from resident peritoneal macrophages from nude mice (Swiss-Webster *nu/nu*). The cells were overlaid with either α-MEM containing 100 ng/ml LPS (×, ●) or with α-MEM alone (△, ○), then challenged with 160 μg/ml zymosan as indicated (×, △).

45 min in a medium containing 50 ng/ml LPS results in a three- to eight-fold enhancement of 20:4 metabolite secretion in response to a variety of triggers.

LPS has multiple effects on mononuclear phagocytes (reviewed in 23). It enhances tumoricidal activity (24, 25), triggers the synthesis and release of tumor necrosis factor (26), hydrolytic enzymes (27, 28), growth factors (29), complement components (30), and prostaglandins (22). Furthermore, while LPS does not trigger the release of reactive oxygen intermediates from macrophages, it does prime macrophages for enhanced secretion of H_2O_2 and O_2^- when subsequently stimulated with PMA (31).

Direct triggering of 20:4 secretion by LPS occurs by a mechanism distinct from that which results in the priming of macrophages for enhanced 20:4 secretion to other stimuli. First, the $K_{1/2}$ for priming is 10 ng/ml LPS, while that for triggering is 500 ng/ml. Second, the cells are fully primed by 50 min, while LPS-induced 20:4 secretion only commences after 90 min. Finally, in work to be reported elsewhere, we show that there are different structural requirements for priming and triggering. The ester-linked 3-OH myristic acid of the lipid A portion of LPS is necessary for triggering but not for priming of 20:4 secretion.

The profile of 20:4 metabolites secreted by macrophages depends on the nature of the stimulus (20, 32). While LPS is capable of potentiating 20:4 metabolite secretion, it does not result in an alteration in the ratio of products released. This suggests that it is the phospholipase activity and not the cyclooxygenase or lipoxygenase pathways that are regulated by LPS treatment.

The mechanism by which LPS primes macrophages for heightened 20:4 release is not clear. LPS might act by increasing the affinity of receptors for their ligands, increasing the number of receptors, or by causing rearangement of receptors on the cell surface. It seems likely, however, that LPS-priming involves steps distal to receptor-ligand binding, since binding and phagocytosis of zymosan, IgG-opsonized erythrocytes, and latex is unchanged in LPS-treated cells (data not

shown). Moreover, LPS appears to act on a step that follows ligation. Previous work (33) showed that triggering of the 20:4 cascade by surface-bound ligands requires Na^+ influx, a protein synthetic step, and a rise in intracellular Ca^{2+}, in that order. PMA and A23187 initiate the cascade at steps following the ligation step. Since LPS changes PMA and A23187 responses, it must affect an event following ligand-receptor binding.

Further evidence that LPS affects an event following receptor ligation comes from kinetic studies. When unprimed cells are stimulated, secretion of 20:4 metabolites commences only after a lag phase. This lag phase is significantly shortened in LPS-treated cells. Treatment with LPS may thus speed and potentiate 20:4 release by establishing one or more preconditions necessary for activation of the phospholipase. Further support for this hypothesis is provided by the ability of LPS to render macrophages responsive to latex particles. Latex particles are ingested by macrophages without the release and metabolism of 20:4 (20 and Fig. 8). However, when LPS-primed cells ingest latex beads, up to 30% of the cellular 20:4 is released as PGI_2 and PGE_2 (Fig. 8). For LPS to be effective, it has to be added to the cells at least 45 min before the latex. This suggests that two categories of signals might be required for 20:4 secretion, priming signals and triggering signals. Zymosan and immune complexes may establish both, latex might be incapable of establishing the priming signals but capable of generating the triggering signals, and ligation of the C3 receptors establishes neither.

There is a precedent for LPS rendering macrophages responsive to a second stimulus. Neither LPS nor latex is capable of stimulating macrophages to secrete plasminogen activator from resident peritoneal cells. However, while macrophages primed by intraperitoneal injection of LPS do not secrete significant amounts of plasminogen activator, they do so when challenged with latex (28).

Little is known about the molecular mechanisms by which LPS might trigger such diverse biological effects. The molecule must first insert into the plasma membrane or bind to specific membrane receptors. It must then interact with enzyme systems involved in cellular signalling. LPS-priming of macrophages for enhanced 20:4 secretion may reflect increased synthesis or induction of phospholipases, or might be due to posttranslational modification of proteins involved in signalling. LPS has been shown (34) to activate the protein kinase C in vitro, and we have shown (unpublished results) that LPS triggers the phosphorylation of a large number of proteins. However, since PMA, which also activates the protein kinase C, does not prime macrophages to secrete 20:4 metabolites in response to latex (data not shown), LPS-induced priming must be more complicated than a mere activation of protein kinase C. We have recently (A. Aderem, E. Pure, and Z. A. Cohn, manuscript in preparation) found that LPS promotes the specific myristoylation of three macrophage proteins with a time and concentration dependence compatible with it having a role in LPS-induced priming. We are currently investigating whether the acylation of proteins involved in celullar regulation promotes their attachment to the plasma membrane and consequently facilitates signal transduction.

Summary

Preincubation of resident peritoneal macrophages with 10–100 ng/ml LPS for 60 min resulted in the cells becoming primed for enhanced (three- to eightfold higher) arachidonic acid (20:4) secretion in response to a variety of triggers. The half-maximal concentration of LPS required for priming was 10 ng/ml irrespective of whether the trigger was particulate (examples: zymosan or immune complexes) or soluble (such as PMA or A23187). Similarly, the time required for half-maximal priming of macrophages was 20 min irrespective of which trigger was used. The primed state persisted for at least 30 h.

LPS-priming of macrophages also affected the kinetics of 20:4 metabolite secretion. The lag phase characteristically observed when 20:4 secretion is triggered was reduced in LPS-primed cells. Furthermore, LPS-primed cells secreted 20:4 metabolites when challenged with latex beads, while unprimed cells did not. These data suggest that stimuli such as zymosan, which elicit 20:4 secretion in macrophages, promote two signals, a priming signal and a triggering signal. LPS is capable of establishing the priming signal but not the triggering signal, while latex promotes the triggering signal but is unable to prime the cells for 20:4 release.

LPS did not effect the profile of 20:4 metabolites secreted in response to any of the triggers, nor did it effect the profile of products synthesized from exogenously added 20:4, suggesting that it did not regulate the 20:4 cascade at the level of either the cyclooxygenase or lipoxygenase pathways.

Macrophages respond to LPS without the intervention of T lymphocytes, since the macrophages from nude mice could be primed for enhanced 20:4 secretion.

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