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Protein Kinase C in Tumoricidal Activation of Mouse Macrophage Cell Lines†

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ABSTRACT: A potential role of protein kinase C (PKC) in lipopolysaccharide- (LPS-) induced tumoricidal activation of macrophages was investigated by using two mouse macrophage cell lines ($P388D_1$ and J774). J774 cells are stimulated by LPS to kill target P815 mastocytoma cells, whereas $P388D_1$ cells fail to develop such an ability. Pretreatment of J774 cells with H-7 or phorbol myristate acetate resulted in a significant inhibition of LPS-induced cytotoxicity, whereas pretreatment with H-8, ML-7, HA1004, or W-7 did not. Since these results suggested a critical role of PKC in the activation process, the properties of PKC in the two cell lines were compared. Western blotting with rabbit antiserum specific for the PKCβ regulatory domain allowed detection of a protein of 79 kilodaltons (kDa) in the detergent lysates of both cell lines that were not stimulated by LPS. However, LPS treatment resulted in the appearance of a second protein of 40 kDa only in J774 cells and not in $P388D_1$ cells. Furthermore, two forms of protein kinase (one basic and the other acidic) were identified in the cytosol of J774 cells by HPLC on DEAE-5PW, whereas only the basic form was found in $P388D_1$ cells. On the basis of the response of the basic and acidic form protein kinases to phosphatidylserine (PS), diolein, and Ca^{2+}, the basic form was found to contain both regulatory and catalytic domains of PKC, whereas the acidic form was suggested to represent the PKC catalytic domain. This was confirmed by Western blotting with the rabbit anti-PKC regulatory domain serum, which whowed the presence of two proteins of 79 and 40 kDa in the basic form kinase of J774 cells. No protein was recognized by this antiserum in the acidic form kinase of J774 cells. To confirm the presence of the catalytic domain in both basic and acidic forms of kinase, J774 cytosols were incubated with [^{3}H]staurosporine (SS) and analyzed by Sephadex G-150 gel filtration, which separated [^{3}H]SS-binding proteins into two major peaks. When the first and second [^{3}H]SS-binding proteins were separately chromatographed on DEAE-5PW, they were eluted with the basic and acidic protein kinase active fractions, respectively. The acidic form of kinase therefore contains the catalytic but not the regulatory domain of PKC. Collectively, these results suggest that proteolytic cleavage of PKC to generate the catalytic domain fragment may serve an important role in LPS-induced tumoricidal activation of macrophages.

Mouse macrophages and macrophage-like cell lines acquire the ability to kill tumor cells and bacteria following the interaction with IFN-γ and/or LPS (Hibbs et al., 1981; Pace & Russell, 1981; Adams, 1989). The LPS-triggered biochemical sequence of events leading to tumoricidal activation of macrophages has been the subject of intense studies. Jakway and DeFranco (1986) suggested that a pertussis toxin sensitive Gi protein, which inhibits adenylate cyclase, may play a critical role in mediating the effects of LPS on $P388D_1$ cells

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or a B cell line (WEHI-231). Prpic et al. (1987) presented evidence that the treatment of thioglycolate-elicited murine peritoneal macrophages with LPS or lipid A promptly triggers the generation of inositol triphosphate (IP_3),[1] indicating LPS-stimulated activation of phosphatidylinositol 4,5-bisphosphate (PIP_2) specific phospholipase C (PLC). Macrophages also respond to LPS by increased synthesis and secretion of various immunoregulatory molecules such as prostaglandins (Jakway & DeFranco, 1986), tumor necrosis factor (Kornbluth & Eddington, 1986), or IL-1 (Dinarello, 1984), suggesting a possible activation of phospholipase A_2 (PLA_2) by LPS as well. Diacylglycerol (DAG), one of the second messengers produced as a result of PLC activation, could be cleaved by DAG lipase to liberate a precursor of prostaglandins (Bell et al., 1979), which in turn activates adenylate cyclase via the specific receptor and Gs protein (Fernandez-Botran & Suzuki, 1984). On the other hand, DAG and Ca^{2+} released from the intracellular storage site by IP_3 action may lead to the activation of protein kinase C (PKC). Thus, the initial target of LPS action could be PLC or PLA_2 and the activation of these enzymes may be followed by the activation of PKC or cAMP-dependent protein kinases (PKA), respectively.

A mouse macrophage-like cell line, J774, acquires, in response to LPS, an ability to kill a TNF-α-insensitive target P815 mastocytoma cell, whereas another cell line, $P388D_1$, does not. We have therefore examined whether or not such a difference in LPS responsiveness could be attributed to the difference in the properties of LPS-triggered PKC. The results presented herein will show that pretreatment of J774 cells with H-7 or phorbol myristate acetate (PMA) effectively blocked LPS-triggered tumoricidal activation, suggesting a participation of PKC in the activation process. Some of the cytosolic PKC of J774 cells appeared to be converted to the catalytic domain fragment (referred to as PKM) upon LPS treatment, whereas that of $P388D_1$ cells was not. Two alternative pathways to activate PKC following LPS treatment of mouse macrophages will be discussed.

Materials and Methods

Cells. The mouse macrophage-like cell lines $P388D_1$ and J774 were derived from methylcholanthrene-induced neoplasm of a DBA/2 mouse and from a tumor of a female BALB/c mouse, respectively. Both cell lines have been shown to possess characteristics typical of macrophages (Koren et al., 1975; Ralph et al., 1975). $P388D_1$ and J774 cells were grown in suspension culture at 37 °C in an atmosphere containing 5% CO_2 in a culture medium consisting of RPMI 1640, heat-inactivated (56 °C, 30 min) FCS (10%), streptomycin (100 μg/mL), penicillin (100 units/mL) (all from Hazelton Dutchland, Denver, PA). Cell density was maintained between approximately 5×10^5 and 1×10^6/mL. Under these culture conditions, the generation time of the two cell lines was about 24 h. The P815 mastocytoma cell line, which originated from a DBA/2 mouse, was obtained from the American Type Culture Collection (Rockville, MD) and was cultured in RPMI 1640 medium containing 10% FCS in a similar manner as macrophages. All of the culture media used as well as the reagents added to the culture were essentially LPS-free (<0.1 ng/mL by the Limulus amebocyte lysate assay).

^{51}Cr-Labeling of P815 Cells. P815 cells were radiolabeled for 1 h at 37 °C with 200 μCi of $^{51}Cr/1 \times 10^7$ cells by using ^{51}Cr-labeled sodium chromate (specific activity of 300–500 mCi/mg; ICN Biochemicals, Irvine, CA). ^{51}Cr-labeled P815 cells were washed once, centrifuged, and allowed to leak for 1 h at 37 °C in complete RPMI 1640 medium with 10% FCS (Johnson & Adams, 1986).

Macrophage Cytotoxicity Assay. Killing of radiolabeled P815 cells was assayed by using a ^{51}Cr release assay (Johnson & Adams, 1986). Briefly, $P388D_1$ or J744 cells (1×10^5 cells/well) were plated in a well of a 96 flat-bottom well tissue culture plate and were cultured for 2 h at 37 °C with 0.2 mL of RPMI 1640 medium containing 10% FCS with or without LPS (10 ng/mL or 10 μg/mL). Supernatants were then aspirated, and monolayers were washed twice with RPMI 1640 medium. ^{51}Cr-labeled target cells (2×10^4/well) were added to each well in 0.2 mL of RPMI 1640 medium containing 10% FCS (effector:target ratio of 10:1). Each treatment was assayed in triplicate. Each experiment was repeated at least three times. Following the coculture for 16 h, an aliquot (0.1 mL) of supernatant was removed from each well and was assayed for radioactivity in a γ counter. Results were expressed as percent specific ^{51}Cr release, as calculated by the following equation:

$$\% \text{ specific } {}^{51}\text{Cr release} = \frac{100(\text{experimental release} - \text{spontaneous release})}{(\text{total releasable counts} - \text{spontaneous release})}$$

Spontaneous release was measured in wells where untreated macrophages were incubated with ^{51}Cr-labeled target cells and culture medium only. Total releasable counts were obtained by incubating ^{51}Cr-labeled P815 target cells with 0.2 mL of 2% Triton X-100.

Partial Separation of PKC. This was carried out by the method described by Fan et al. (1988) with a substantial reduction in the concentration of EDTA and EGTA used in buffer B, as follows. Briefly, J774 or $P388D_1$ cells (2×10^7/group) were cultured for 0–6 h with or without LPS (1 μg/mL) in RPMI 1640 medium containing FCS, penicillin, and streptomycin. At the end of the incubation period, the cells were washed with ice-cold buffer A (5 mM Tris-HCl, pH 7.5, 5 mM NaCl, 0.5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), and 10 μg of leupeptin/mL), resuspended in 4 mL of buffer A, and homogenized. The homogenates were centrifuged for 1 h at 100000*g* at 4 °C. The supernatant (1 mL) was applied at 4 °C with the use of HPLC to a column of DEAE-5PW (7.5 × 75 mm) (Beckman) or DE52 (Whatman) (bed volume of 6 mL) previously equilibrated against buffer B (20 mM Tris-HCl, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, and 2 mM DTT). Proteins were first eluted at 1 mL/min with 30 mL of Buffer B and then with an additional 30 mL of buffer B containing 50 mM NaCl, and a total of 60 fractions containing 1 mL each were collected. PKC activity was assayed within 24 h after the fractionation, as follows.

PKC Assay. An aliquot (100 μL) of each fraction from DEAE-5PW or DE52 column chromatography was mixed with 100 μL of the PKC assay cocktail (20 mM Hepes/NaOH buffer, pH 7.5, containing 10 mM $MgCl_2$, 5 mM Ca^{2+}, 120 μg of PS/mL, 12 μg of diolein/mL, 5 mM DTT, 50 μM ATP, and 1 mg of lysine-rich histone type III/mL; all reagents from Sigma). The reaction was initiated by adding 2 μCi of [γ-^{32}P]ATP (2–10 Ci/mmol; New England Nuclear) to the reaction mixture and was carried out at 30 °C for 20 min. At

[1] Abbreviations: DAG, 1,2-diacylglycerol; DTT, dithiothreitol; FCS, fetal calf serum; IFN, interferon; IL, interleukin; IP_3, inositol 1,4,5-triphosphate; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKM, catalytic domain fragment of protein kinase C; PLA_2 and PLC, phospholipases A_2 and C, respectively; PMA phorbol myristate acetate; PS, phosphatidylserine; SS, staurosporine; TNF, tumor necrosis factor.

the end of the reaction, an aliquot (20 μL) of the mixture was spotted on a paper square (2 × 2 cm, Whatman P81 phosphocellulose paper). The paper square was washed first with 5% TCA, then with ethanol, dried, and counted with a β counter.

Antisera. Rabbit antiserum directed against the C-terminal end of the regulatory domain of bovine PKC (residues 280–292) was kindly provided by Dr. P. Parker of the Ludwig Institute for Cancer Research, London, England. Rabbit antiserum directed against the bovine PKCβ C-terminal peptide with the sequence of FVNSEFLKPEVKS (denoted as F13S) was produced at Calico Biological Inc. (Reemstone, PA), as follows. The F13S peptide synthesized by Dr. Crabb of the Alton Jones Cell Science Center (Lake Placid, NY) was covalently coupled to Affi-gel (Bio-Rad). Rabbits were given intramuscularly about 2 mg of Affi-gel-coupled peptide emulsified in complete Freund's adjuvant. They were boosted four times at 10-day intervals with 0.5 mg of peptides emulsified in incomplete Freund's adjuvant. The preimmune sera and antisera were screened by a standard ELISA for the presence of specific antibody. Antiserum containing high titer (1:>5000) of specific antibody was subjected to affinity chromatography on a column of Sepharose 4B coupled to F13S peptide. The antibody bound to the column was eluted, concentrated, and dialyzed against PBS.

Two-Dimensional (2-D) Gel Electrophoresis. This was carried out by the method described by O'Farrell [1975]. J774 or P388D_1 cells (1 × 10^7/experiment) were incubated for 3 h at 37 °C with or without LPS (1 μg/mL). They were then separately washed three times with PBS and lysed by treatment with 1 mL of the lysis solution consisting of 9.5 M urea, 2% NP-40, 2.5% ampholine (Pharmalyte, pH 3.5–10, Pharmacia, Piscataway, NJ), and 1 M 2-mercaptoethanol. The lysate (100 μL) was loaded on first-dimension tube gels (2.5 × 125 mm) containing 3.8% acrylamide, 0.2% bisacrylamide, 9.5 M urea, 2% NP-40, and 2.5% Pharmalyte (pH 3.5–10). Isoelectric focusing was carried out for a total of 7000 volt·h. The gel was then equilibrated against SDS sample buffer and subjected to second-dimension SDS–PAGE in a 12% polyacrylamide gel.

Western Blotting. The proteins separated by 2-D gel electrophoresis or by standard single-dimensional SDS–PAGE were electrophoretically transferred at 4 °C to nitrocellulose membrane (0.45-μm pore, Hoeffer Scientific, San Francisco, CA) with the use of a Transphor TE42 apparatus for 16 h at 250 mA. The membranes were then immersed for 1 h at 25 °C in PBS containing 5% nonfat dry milk, washed three times with PBS, and incubated for 1 h at 25 °C with rabbit antiserum directed against the C-terminal end of the regulatory domain of bovine PKC. The membranes were then washed three times with PBS containing 0.05% Tween 20 and twice with PBS and incubated for 1 h at 25 °C with alkaline phosphatase tagged goat antibody against rabbit IgG (Hy-Clone, Logan, UT). The membranes were then washed four times with Tris-HCl-buffered saline containing 0.3% Tween 20 and four more times with 100 mM Tris-HCl buffer, pH 8.8, containing 1 mM Mg^{2+} and developed by exposure to the substrate nitroblue tetrazolium (0.1 mg/mL)(Fisher, St. Louis, MO) in the presence of 5-bromo-4-chloro-3-indolyl phosphate (5 mg/mL)(Sigma).

[^{3}H]Staurosporine (SS) Binding Assay. J774 or P388D_1 cells (1 × 10^8/experiment) were incubated for 3 h at 37 °C with or without LPS (1 μg/mL), washed with ice-cold buffer A, homogenized in 3 mL of buffer A, and centrifuged for 1 h at 100000*g* at 4 °C. The cytosol fraction (2 mL) was divided into two equal parts. Each cytosolic fraction was incubated for 60 min at 4 °C with 2 μCi of [^{3}H]SS (160 Ci/mmol, New England Nuclear, Boston, MA) in the presence or absence of a 1000-fold molar excess of unlabeled SS. At the end of the incubation period, an aliquot (500 μL) of the cytosol was subjected to gel filtration with the use of a column of Sephadex G-150 (a bed volume of 90 mL), which was previously equilibrated against buffer B. Proteins were eluted with buffer B in a total of 200 fractions (0.5 mL/fraction). The radioactivity in an aliquot (100 μL) of each fraction was measured by a β counter.

Table I: LPS-Induced Cytotoxicity of J774 and P388D_1 Cells[a]

expt	concn of LPS (μg/mL)	% cytotoxicity J774	% cytotoxicity P388D_1
1	0	−0.6 ± 3.9	11.2 ± 4.0
	0.01	55.9 ± 3.8	−9.2 ± 3.4
	10	64.0 ± 5.5	−3.0 ± 5.1
2	0	5.2 ± 3.1	nd[b]
	0.01	39.2 ± 6.9	nd
	10	47.3 ± 2.2	nd
3	0	−8.0 ± 3.0	5.0 ± 3.4
	10	30.0 ± 4.0	0.7 ± 2.9

[a] The cytotoxicity was assayed by measuring the specific release of ^{51}Cr from the ^{51}Cr-labeled target P815 cells following 16-h culture with effector cells at an effector:target cell ratio of 10:1 in the absence or presence of LPS. The total releasable and spontaneously released radioactivity was 4030 ± 120 cpm and 1780 ± 50 cpm (44%) in experiment 1, 4040 ± 220 cpm and 2000 ± 50 cpm (50%) in experiment 2, and 7200 ± 380 cpm and 2780 ± 60 cpm (39%) in experiment 3. Each value represents the mean ±SD of triplicate determinations. [b] nd, not determined.

Results

LPS-Triggered Cytotoxicity. J774 and P388D_1 cells share typical macrophage characteristics such as adherence to glass or plastic surfaces, the expression of Fcγ receptors, phagocytosis of opsonized particles, and the capacity to produce IL-1 and TNF-α. Because they were derived from different tumor cells and originated in different mice, however, they may differ in some other macrophage functions. Comparative biochemical analysis of those particular functions may provide valuable information on the regulatory mechanisms of macrophage function. We have therefore first compared LPS-triggered cytotoxicity of the two cell lines, using the TNF-α-insensitive P815 cells as target. As summarized by Table I, in three separate experiments, the treatment of J774 cells with LPS at 10 ng/mL or 10 μg/mL invariably activated these cells for specific killing of 30–64% of P815 cells. On the other hand, LPS treatment of P388D_1 cells in two separate experiments failed to stimulate them for tumoricidal activity against P815 cells.

Effects of Protein Kinase Inhibitors on Tumoricidal Activation of J774 Cells. In order to examine whether or not PKC or other protein kinases might play a role in LPS-triggered tumoricidal activation of J774 cells, the effects of various protein kinase inhibitors were examined. Pretreatment of J774 cells for 2 h with H-7 [a PKC inhibitor with $K_i \sim 5$ μM (Kawamoto & Hidaka, 1984)], clearly blocked LPS-induced cytotoxicity in a dose-dependent manner, from about 63% killing in the absence of the inhibitor to 20% killing at 25 μM (68% inhibition). The lowest concentration of H-7 that gave a statistically significant inhibition (33%) was close to the K_i value of 5 μM, suggesting a probable importance of activation of PKC in LPS-induced tumoricidal activity of J774 cells. However, since H-7 is not exclusively specific for PKC (Shibanuma et al., 1987), the effects of PMA on LPS-induced tumoricidal activity of J774 cells were also studied. Prein-

cubation of J774 cells with PMA for 3 h was found to also block, in a dose-dependent manner, LPS-induced cytotoxicity. The concentration of PMA causing 50% inhibition was between 10 and 100 nM. At all concentrations tested, neither H-7 nor PMA was toxic to J774 cells, as judged by their viability, or to P815 cells, as judged by the degree of spontaneous release of ^{51}Cr.

The effects of inhibitors of PKA (H-8), calmodulin-dependent kinase (W-7), and myosin light chain kinase (ML-7) and of HA1004 were next investigated. Among these, only H-8 significantly inhibited LPS-induced J774 cell activation, although a relatively high concentration (>33 μM) was required to exert a significant suppression (no inhibition at 33 μM and 75% inhibition at 66 μM). The inhibitory effect of H-8 may thus be due to the suppression of PKC rather than PKA, because the K_i of this inhibitor for PKC is in the range of 15 μM (Hagiwara et al., 1987). The other protein kinase inhibitor, HA1004, also reduced LPS-induced cytotoxicity of J774 cells to a small extent (28% inhibition) at the concentration >50 μM. Neither W-7 nor ML-7 exerted any inhibitory effects at concentrations up to 10 μM. Higher concentrations of W-7 or ML-7 could not be tested because of their toxicity toward J774 cells.

Thus, the data with the use of various protein kinase inhibitors suggested that, among protein kinases examined, activation of PKC plays probably an important role for the development of LPS-triggered tumoricidal activity of J774 cells. However, the question was raised as to whether the noted inhibitory effect of H-7 is due indeed to suppression of PKC, since H-7 is shown to be not exclusively specific for PKC, although the noted inhibitory effects of PMA are probably more specifically due to depletion of PKC (Nishizuka, 1986). To verify whether or not PMA pretreatment indeed depletes PKC, changes in PKC activity and quantity following PMA treatment of J774 cells were investigated. To this end, J774 cells (1×10^8) were incubated for 3 h at 37 °C with or without 10 or 100 nM PMA. PMA-treated or untreated J774 cells were homogenized in buffer A. Following centrifugation of the homogenates, the cytosol fractions were separately fractionated by DE52 chromatography, as described under Materials and Methods. PKC assay of the fractions eluted by buffer A containing 50 mM NaCl showed that treatment of J774 cells with 10 and 100 nM PMA decreased PS, diolein, and Ca^{2+}-dependent protein kinase activities to 0.56 and 0 nmol mg^{-1} min^{-1}, respectively, from the activity found in the cytosol of PMA-untreated cells of 1.58 nmol mg^{-1} min^{-1}. The quantitative changes in PKC due to PMA treatment were then investigated by Western blot, by using antibody directed against the synthetic C-terminal peptide (F13S) of bovine PKCβ. To this end, equal amounts (50 μg each) of the cytosols prepared as above from untreated and PMA-treated J774 cells were subjected to SDS–PAGE. The proteins separated were electrophoretically transferred to nitrocellulose membranes and incubated with anti-F13S antibody as described under Materials and Methods. As shown by Figure 1, the intensity of the signal from the 79-kDa protein recognized by this antibody decreased following treatment with 10 nM PMA and almost completely disappeared upon treatment with 100 nM PMA. An experiment with the use of Triton X-100 lysates, instead of the cytosols, of untreated and PMA-treated J774 cells gave a similar result, showing a dose-related decrease of PKC upon PMA treatment, suggesting that the PMA-induced decrease of PKC from the cytosol is not due to the translocation of the enzyme to the membrane. These data thus strongly indicated that PMA treatment of J774 cells indeed results in the depletion of PKC from the cytosol, which may contribute to the LPS unresponsiveness following PMA treatment.

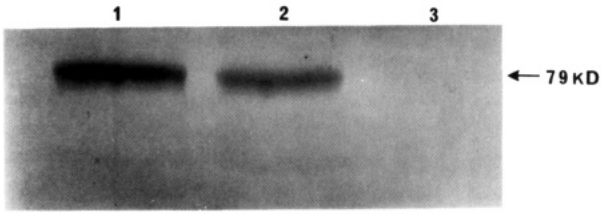

FIGURE 1: Western blotting of the cytosols of untreated and PMA-treated J774 cells. The cytosols were prepared from J774 cells that were incubated for 3 h at 37 °C without (lane 1) or with 10 nM (lane 2) or 100 nM (lane 3) PMA. An aliquot containing 50 μg of proteins from each cytosol was subjected to SDS–PAGE. The proteins were then electrophoretically transferred onto nitrocellulose membranes and incubated with rabbit antiserum directed against the synthetic peptide F13S. The arrow indicates the molecular mass (79 kDa) of the protein recognized by the antiserum. The additional area of the membrane other than the 79-kDa bands did not contain other proteins recognized by this antiserum and was cut out from the picture to save space. An additional experiment carried out with the use of Triton X-100 lysates of PMA-treated and untreated J774 cells gave essentially the same Western blotting result.

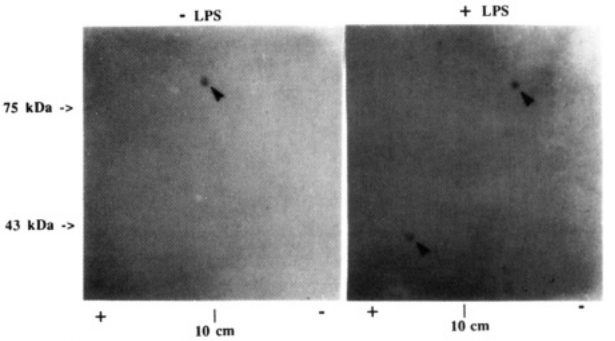

FIGURE 2: Western blotting of the cytosols of untreated and LPS-treated J774 cells. The cytosols were prepared from LPS-treated and untreated cells and were subjected to 2-D gel electrophoresis. The proteins were then electrophoretically transferred onto nitrocellulose membranes and blotted with rabbit antiserum directed against the C-terminal end (residues 280–292) of the regulatory domain of bovine PKC, as described under Materials and Methods. Arrows indicate the proteins recognized by the antiserum. Distance (10 cm) from the cathode is marked at the bottom to facilitate the comparison. The area not shown in the picture did not contain any other protein spot. The results with untreated and LPS-treated P388D_1 cells were identical with that from untreated J774 cells and are therefore not shown.

Comparison of PKCs of the Two Cell Lines. The inhibition of LPS-induced tumoricidal activation of J774 cells by pretreatment with either H-7 or PMA suggested the involvement of PKC in the macrophage activation process. The question raised was whether or not the difference in the response to LPS between J774 and P388D_1 cells might be a reflection of a difference in the properties of PKCs between the two cell lines. To examine this question, the detergent lysates of both cell lines were separately prepared from the LPS-treated and untreated two cell lines. They were subjected first to IEF and then to SDS–PAGE in the second dimension. Proteins on 2-D gels were separately transferred to nitrocellulose membranes and were probed with rabbit anti-regulatory domain serum directed against the C-terminal end of the regulatory domain of bovine brain PKCβ (Parker et al., 1986). This antiserum recognized a single 79-kDa protein present in the lysates of both cell lines not treated with LPS. As shown by Figure 2, the lysate of LPS-treated J774 cells was found to contain an additional 40-kDa protein recognized by the antiserum, whereas the lysate of LPS-treated P388D_1 cells did not contain this 40-kDa protein.

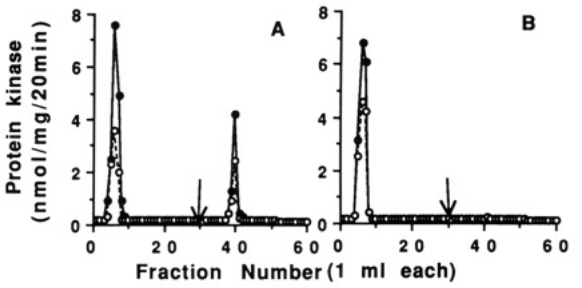

FIGURE 3: Protein kinase activities of the cytosols of untreated (open circle) and LPS-treated (closed circle) J774 (A) and P388D$_1$ (B) cells that were fractionated by HPLC on a column of DEAE-5PW, as described under Materials and Methods.

Furthermore, as illustrated by Figure 3A, the protein kinase assay of the cytosol of LPS-unstimulated J774 cells fractionated by HPLC on a column of DEAE-5PW clearly showed the presence of two protein kinase active fractions (basic peak I between the fraction numbers 5 and 9 and acidic peak II between the fraction numbers 38 and 40), whereas that of unstimulated P388D$_1$ cells showed only one peak corresponding to the basic form of J774 cells (Figure 3B). LPS treatment for 3 h enhanced the basic form kinase activity present in the cytosols of both J774 and P388D$_1$ cells about 1.5–2-fold. The acidic form kinase activity present in J774 cells also increased about 1.5-fold upon LPS treatment. LPS treatment of P388D$_1$ cells did not generate this acidic form protein kinase activity. Time course studies showed that LPS-induced enhancement of protein kinase activities in both peak I and II of J774 cells and in peak I of P388D$_1$ cells was maximal at 3 h following LPS treatment.

In order to investigate whether or not protein kinase active peaks I and II of J774 cells indeed contain PKC, peak I and II fractions and the fractions from 10 through 37, which were separated by DEAE-5PW HPLC of the cytosol of LPS-treated J774 cells, were separately concentrated by ultrafiltration through a PM10 membrane and subjected to SDS–PAGE. The proteins separated were transferred electrophoretically to nitrocellulose membranes, and the blots were incubated with rabbit anti-PKCβ regulatory domain serum. As shown by Figure 4, peak I (lane 1) clearly contained a 79-kDa and a 40-kDa protein, whereas peak II (lane 3) or the fraction between two kinase active fractions (lane 2) did not contain any protein recognized by this antiserum. These results thus suggested that peak I of J774 cells contains both intact PKC and a regulatory domain fragment of 40 kDa and that peak II protein kinase may represent a catalytic domain fragment. If this is the case, protein kinase activity of peak I should be up-regulated by PKC regulatory molecules (PS, diolein, and Ca^{2+}), whereas that of peak II should not be affected by the presence of these regulatory molecules. Indeed, the enzymatic activity of peak I obtained from LPS-treated or untreated J774 cells assayed in the presence of PS (60 μg/mL), diolein (6 μg/mL), and Ca^{2+} (0.5 mM) were found to be about 38% and 42%, respectively, higher than those assayed in the presence of EGTA (0.5 mM) ($p < 0.05$). On the other hand, the enzymatic activity of peak II obtained from both LPS-treated and untreated J774 cells was not significantly affected by these regulatory molecules of PKC ($p > 0.5$).

[^{3}H]Staurosporine-Binding Studies. The above results suggested that basic (peak I) PKCs in both LPS-activatable J774 and nonactivatable P388D$_1$ cells may be activated in response to LPS treatment. Some of the PKCs in J774 cells appeared to be proteolytically cleaved to generate the catalytically active fragment, whereas PKCs in P388D$_1$ cells were not. The question is whether or not the acidic kinase active

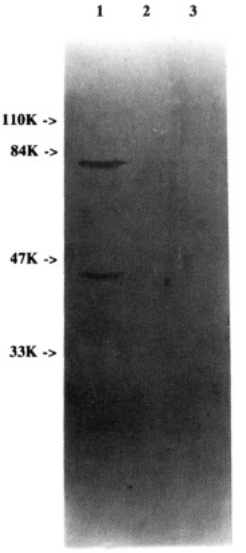

FIGURE 4: Western blotting of protein kinase active peak I (lane 1) and II (lane 3) fractions and the fractions between I and II (lane 2) of LPS-treated J774 cytosol separated by HPLC with the use of DEAE-5PW column as in Figure 3. The protein kinase active fractions 5–9 (peak I) (lane 1) and 38–40 (peak II) (lane 3) and the control fractions 10–35 (lane 2) were separately pooled, concentrated by ultrafiltration through PM10 membranes, subjected to SDS–PAGE, electrophoretically transferred to nitrocellulose membranes, and probed with the rabbit anti-regulatory domain serum used in Figure 2.

fraction (peak II) of J774 cells indeed contains the catalytic domain of PKC. This question was investigated with the use of [^{3}H]staurosporine (SS), which should bind with high affinity ($K_D \sim 2$–4 nM) to the catalytic domain of PKCs (Gross et al., 1990), as follows. The cytosol fraction (2 mL) in buffer A was obtained from J774 cells (1×10^8) that were treated with LPS (1 μg/mL) for 3 h and divided into two equal parts. They were incubated with 2 μCi of [^{3}H]SS for 30 min at 4 °C in the presence or absence of a 1000-fold molar excess of unlabeled SS. An aliquot (0.5 mL) of each cytosol was then separately subjected to gel filtration over a Sephadex G-150 column (bed volume of 90 mL) previously equilibrated against buffer B. Proteins were eluted with buffer B, and 200 fractions containing 0.5 mL each of the eluates were collected. The radioactivity in an aliquot of 200 μL from each fraction was measured. As depicted by Figure 5, the gel filtration of the cytosol incubated with [^{3}H]SS in the absence of excess unlabeled SS resulted in the separation of two ill-defined radioactive peaks designated as peak A (fraction numbers 51–61) and B (62–120) and one well-defined peak designated as C (121–180). The cytosol incubated with [^{3}H]SS in the presence of a 1000-fold molar excess of unlabeled SS gave rise to only one radioactive peak eluted between fraction numbers 120 and 180. These results suggested that the radioactivity in peaks A and B represents [^{3}H]SS bound to proteins, whereas that in peak C represents protein-unbound [^{3}H]SS. Indeed, almost all (>95%) of the radioactivity in peaks A and B was precipitated by 5% TCA, whereas almost all (>95%) of the radioactivity in peak C was not. The binding of [^{3}H]SS to proteins must be specific, because the presence of a large excess of unlabeled SS totally blocked radiolabeling of proteins in peaks A and B. [^{3}H]SS-bound proteins in peaks A and B were then separately concentrated by ultrafiltration through a PM10 membrane and separately subjected to HPLC on a column of DEAE-5PW as described above for fractionation of protein kinases. The measurement of the radioactivity of each fraction gave a result shown by Figure 6. Peak A fraction was found to be separated into two basic radioactive peaks (maximum

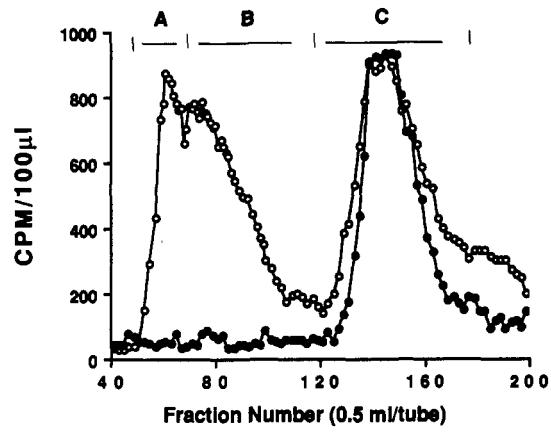

FIGURE 5: Gel filtration of [^{3}H]SS-bound cytosol of LPS-treated J774 cells. J774 cells (1×10^8) were treated at 37 °C for 3 h with LPS (1 µg/mL), homogenized in 3 mL of buffer A, and centrifuged. The resultant cytosol (2 mL) was divided into two equal parts. They were incubated for 30 min at 4 °C with [^{3}H]SS (2 µCi) in the presence (closed circles) or absence (open circles) of a 1000-fold molar excess of unlabeled SS. An aliquot (0.5 mL) of each cytosol was then separately passed through a column (bed volume of 90 mL) of Sephadex G-150 previously equilibrated against buffer B. The two radioactive fractions (from numbers 51 to 61 and from 62 to 120) obtained from the cytosol incubated with [^{3}H]SS in the absence of excess unlabeled SS were separately pooled as peaks A and B, respectively. Most (>95%) of the radioactivity in these two peaks was precipitated with 5% TCA.

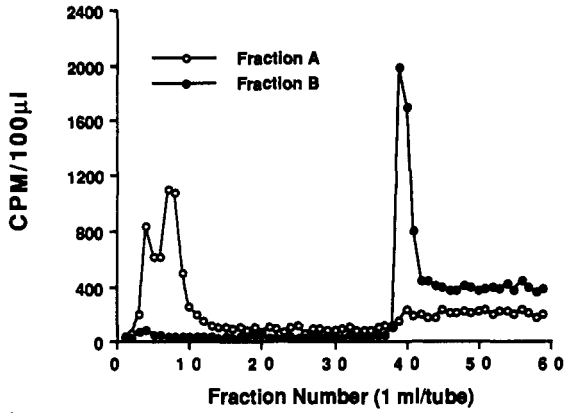

FIGURE 6: HPLC of [^{3}H]SS-bound cytosolic fractions on DEAE-5PW. Peaks A (open circles) and B (closed circles) obtained by gel filtration of [^{3}H]SS-bound cytosol of LPS-treated J774 cells as in Figure 5 were separately concentrated by ultrafiltration through a PM10 membrane and were subjected to HPLC as in Figure 3.

at the fraction numbers 4 and 7), whereas peak B fraction was eluted as a single peak at fraction number 39–41. The elution position of these distinct radioactive peaks clearly coincided with peaks I and II protein kinase active fractions depicted in Figure 3. A similar experiment with LPS-treated P388D$_1$ cells gave a single radioactive peak that was eluted in a fraction corresponding to peak I protein kinase active fraction and no radioactive peak corresponding to peak II of J774 cells. These results thus indicated that both protein kinase active peaks I and II separated by DEAE-5PW HPLC of the cytosol of J774 cells can specifically bind to [^{3}H]SS, suggesting the presence of the PKC catalytic domain in each peak.

Discussion

Several studies have recently suggested that LPS-triggered activation of PIP_2-specific PLC may be an early, critical biochemical event during macrophage activation. Evidence that the treatment of mouse peritoneal macrophages with LPS or lipid A promptly leads to activation of PIP_2-specific PLC has been presented by Prpic et al. (1987). In their studies, the activation of PLC, as measured by the formation IP_3, was rapid in time but rather modest in extent. IP_3 formed in response to LPS treatment was shown to reach a plateau within 1 min at a maximal change of about 130% from the time zero value. LPS-stimulated activation of PLC was also noted in our recent studies of the two macrophage cell lines (Chang et al., 1990) and thus partly confirmed the results of Prpic et al. Thus, the data from two laboratories independently showed that LPS treatment of macrophages led to the modest activation of PIP_2-specific PLC within 1–20 min. However, a critical question remains as to whether or not LPS-triggered activation of PLC is a primary inducer for tumoricidal activation of macrophages. LPS-stimulated activation of PLC would produce two different types of second messengers: 1,2-DAG and IP_3 from PIP_2 (Pirpic et al., 1986). DAG and Ca^{2+} released from the intracellular storage site by the action of IP_3 would activate PKC (Nishizuka, 1986, 1988). Weiel et al. (1986) earlier suggested probable activation of PKC following stimulation of mouse macrophages by LPS because of the similarity of LPS-induced phosphorylation pattern of cellular proteins to that induced by PMA.

The finding of the inhibition of LPS-triggered cytotoxicity by pretreatment of LPS-activatable J774 cells with a PKC inhibitor, H-7 (Kawamoto & Hidaka, 1984), suggests a probable involvement of PKC activation in LPS-triggered tumoricidal activation of J774 cells. The roles of other kinases such as myosin light chain kinase or calmodulin-dependent kinase in tumoricidal activation appeared to be limited, since the inhibitors of these enzymes, ML-7 or W-7, failed to block LPS-triggered macrophage activation. Similarly, PKA activation seemed to play a relatively less significant role in macrophage activation, since only relatively large doses of H-8 (>33 µM) were found to be required for significant inhibition of tumoricidal activity. The noted inhibitory action of H-8 could be due to the suppression of PKC rather than PKA, because the K_i of this inhibitor for PKC is in the range of 15 µM (Hagiwara et al., 1987). In this regard, it is interesting to note that tumoricidal activation of macrophages induced by IFN-γ or IFN-β has been shown to be blocked also by inhibitors of PKC, such as H-7 (Celada & Schreiber, 1986; Radioch & Varesio, 1988). The results with the use of inhibitors of various kinases thus suggested a potential importance of the activation of PKC in LPS-triggered tumoricidal activation of J774 cells. However, because neither H-7 nor H-8 is strictly specific for PKC, a possibility remains that these lipophilic inhibitory molecules may interact with LPS and thus block LPS action. Although the finding of the inhibition of tumoricidal activation by PMA pretreatment, which depletes PKC from the cell (Figure 1), also suggested an importance of PKC activation in tumoricidal activation, this result must be cautiously interpreted, because changes in PKC status during PMA pretreatment may have altered various cellular properties that may have resulted in the loss of LPS-triggered macrophage activation.

PKC has been shown to be involved in a wide variety of cellular functions, such as secretion and exocytosis, modulation of ion conductance, regulation of receptor interaction with components of the signal transduction apparatus, smooth muscle contraction, gene expression, and cell proliferation (Nishizuka, 1986, 1988). PKCs in various tissues are polymorphic, and seven isoenzymes denoted as α, βI and βII, γ, δ, ϵ, and ζ have so far been identified (Ono et al., 1988; Coussens et al., 1986). Gene sequence analysis of these isoenzymes suggests a great degree of sequence homology, not only among themselves but also with many other protein ki-

nases. The N-terminal half of the single-chain 80-kDa PKC is considered to be the regulatory domain and contains the sites involved in binding of PKC regulatory molecules such as PS, Ca^{2+}, and 1,2-DAG. The C-terminal half of the enzyme contains two conserved regions considered to be the catalytic domain. The site that is susceptible to the Ca^{2+}-dependent neutral protease calpain (Suzuki et al., 1987; Kishimoto et al., 1989) has been shown to be within the third variable region, which separates the regulatory and catalytic domains.

The critical question is whether LPS treatment leads to activation of macrophage PKC only through generation of DAG and IP_3 or also through an alternative pathway that might involve the calpain/calpastatin system. Both LPS-activatable J774 cells and nonactivatable $P388D_1$ cells possess in their cytosol, before LPS treatment, PKC of 79 kDa recognized by an antibody directed against the regulatory domain of bovine brain PKCβ. The data of Figures 2–6 collectively suggest that, upon LPS treatment, some of the PKC in J774 cells is cleaved to generate the regulatory and catalytic domain fragments, whereas PKC in $P388D_1$ cells remained intact. Evidence supporting this concept includes the following: (1) the identification of an additional 40-kDa protein by anti-PKC regulatory domain antibody in LPS-treated J744 cells but not in LPS-treated $P388D_1$ cells (Figure 2), (2) the presence of basic and acidic forms of protein kinase activity in J774 cells separable by DEAE-5PW HPLC (Figure 3A), and (3) the presence of only the basic form of protein kinase activity in $P388D_1$ cells (Figure 3B). The data of Figures 4–6 suggest that the basic form of protein kinase of J774 cells contains both regulatory and catalytic domains of PKC, whereas the acidic form of protein kinase contains only the catalytic domain. Evidence supporting this concept includes the following: (1) the presence of both intact PKC of 79 kDa and a regulatory domain fragment of 40 kDa in the basic form of protein kinase (peak I) of LPS-treated J774 cells (Figure 4), (2) the absence of regulatory domain in the acidic form (peak II) of protein kinase of J774 cells (Figure 4), (3) the up-regulation of the basic form, but not the acidic form, of protein kinase activity by the presence of PS, diolein, and Ca^{2+}, and (4) the specific binding of [^{3}H]SS, a specific inhibitor of PKC (Gross et al., 1991), to both basic and acidic forms of protein kinase of J774 cells (Figures 5 and 6). However, further experimentation is needed to definitively prove the concept that the acidic form of protein kinase of J774 cells indeed contains the catalytic domain fragment. In addition, a possibility exists that the differences other than PKC phenotype between the two cell lines may be more relevant to LPS-triggered tumoricidal activation, because the two cell lines originated from different sources and have been in culture for many years.

Thus, LPS treatment of macrophages may lead to activation of PKC by two distinct pathways: (1) via a classical DAG–IP_3-mediated pathway that occurs in both LPS-activatable and non-activatable cell lines and (2) via the activation of Ca^{2+}-dependent neutral protease (calpain) in LPS-activatable cells, which cleaves PKC to generate PKM, the activity of which does not require regulatory molecules such as DAG, Ca^{2+}, and phospholipid. The lack of generation of 40-kDa protein recognizable by anti-regulatory domain antibody in LPS-nonactivatable $P388D_1$ cells could be due either to structural alteration of the V_3 region of PKC, which renders PKC resistant to calpain action, or to the difference in properties of $P388D_1$ calpain from those of J774 calpain. Although there is no direct evidence at present which indicate that LPS activates calpain, it is interesting to consider this as a possible pathway, particularly because our recent preliminary studies demonstrated a dose-dependent inhibition of LPS-triggered tumoricidal activation of J774 cells by a calpain inhibitor, E64 [1-[L-*N*-(*trans*-epoxysuccinyl)leucyl]amido-4-guanidinobutane (Barrett et al., 1982)] (manuscript in preparation).

Evidence that calpain may regulate the activity of PKC has been presented by many laboratories (Tapley & Murray, 1985; Murray et al., 1987; Pontremoli et al., 1986). Incubation of platelets or neutrophils with PMA was shown to result in the formation of a 50-kDa PKM, active in the absence of added Ca^{2+} and phospholipid, which eluted from DE52 cellulose at a higher salt concentration than PKC. Formation of this kinase was blocked by preincubation of permeabilized platelets with leupeptin, a calpain inhibitor (Murray et al., 1987). The cleavage of PKC to PKM by calpain frees the enzyme from its regulatory constraint and releases active kinase (PKM) into the cytosol. Thus, PKM would have access to a different range of substrates than membrane-bound PKC. Little is known, however, as to whether or not PKM and PKC indeed catalyze phosphorylation of different sets of cellular proteins, which can be related to the regulation of gene expression necessary for tumoricidal activation of macrophages.

Various interesting avenues of exploration are suggested by the present studies, some of which are being investigated in our laboratory. These include determining whether the noted differences in the properties of PKC between LPS-activatable and nonactivatable cells are due to the structural difference of PKCs or to the differences in the properties of calpain or calpastatin. The sites susceptible to calpain action have been localized within the V_3 region of PKC. The noted lack of generation of either regulatory domain fragment or acidic form of protein kinase by LPS treatment of $P388D_1$ cells could be due to the alteration of the amino acid sequence of this region of PKC of $P388D_1$ cells, which makes this region resistant to the calpain action. Another important question is whether or not LPS actually activates calpain and leads to the generation of PKM. Calpain consists of two subunits, the 80-kDa and 30-kDa proteins (Emori et al., 1986a,b). Upon autolysis, the 80-kDa calpain subunit becomes the protease-active 76–78-kDa protein. The N-terminal, glycine-rich hydrophobic domain of the 30-kDa subunit has been shown to interact with phospholipids and biological membranes (Imajoh et al., 1986) as well as galactosyl residue containing polysaccharide (Zimmerman & Sclaepfer, 1988). LPS is expected to interact with membranes through its hydrophobic lipid A moiety, and there is now good photoaffinity labeling evidence for the presence of 80-kDa LPS receptor protein in the membrane of lymphocytes and macrophages (Lei & Morrison, 1988a,b). Thus, LPS may interact, through its polysaccharide component, with the N-terminal regulatory domain of the 30-kDa calpain subunit, which together with the 80-kDa subunit is associated with the inner side of the plasma membrane. The interaction between 30-kDa calpain subunit and LPS may photoaffinity label 80-kDa protein and promote autolysis of 80-kDa subunit to produce proteolytically active 76–78-kDa calpain fragment. This type of calpain activation has been observed by Zimmerman and Schlaepfer (1988) during the binding of calpain to agarose matrix.

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Registry No. Protein kinase, 9026-43-1; calpain, 78990-62-2.

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