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LPS−induced activation of phospholipase A_2 phospholipase C and protein kinase C of murine macrophage−like cell lines (J774 and P388D1) [1]

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ABSTRACT

A murine macrophage−like cell line, J774, acquired, in response to LPS, an ability to kill tumor necrosis factor (TNF)−insensitive target P815 mastocytoma cells, whereas another cell line, P388D1, did not. LPS−triggered signaling mechanisms between the two cell lines were compared with an aim to inquire about the possible nature of the above-mentioned difference. The results showed that two cell lines respond to LPS−treatment by parallel activation of both phospholipases C and A_2 (PLC and PLA_2) to approximately the same extent. The maximum response of both enzymes of J774 cells was noted within 10 min of the treatment, whereas that of P388D1 cells required more than 20 min. The other properties of LPS-responsive enzymes studied were similar between two cell lines, including

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Abbreviations used: AC, adenylate cyclase; cAMP, adenosine 3'-5' cyclic monophosphate; CT, cholera toxin; DAG, 1, 2−diacylglyceride; DEX, dexamethasome: DTT, dithiothreitol; Gi, GP, and Gs proteins, guanine nucleotide−binding regulatory proteins; GTPγs, guanosine 5'[γ-thio] triphosphate; HBSS−LH, Hank's balanced salt solution supplemneted with LiCl: and Hepes; IP_3, inositol−1, 4, 5−triphosphate; NM, neomycin; pBPB, p−bromophenacyl bromide; PC, PE, PI, and PS, phosphatidyl−choline,−ethanclamine, −inositol, and−serine, respectively; PIP_2, phosphatidylinositol−4, 5−bisphosphate; PG, prostaglandin; PLA_2 and PLC, phospholipases A_2 and C, respectively; PT, pertussis toxin; TLC, thin layer chromatography. PKC, protein kinase C; EDTA, ethyleneglycol−bis−(beta-aminoethylether)N.N, N', N'−tetraacetic acid.

Ca^{2+} augmentation of enzyme activation, participation of guanine nucleotide binding (G) proteins in the initial activation processes, and inhibition of enzyme activation by the prior treatment of cells with cholera or pertussis toxins etc. Moreover, LPS−triggered activation of PLC and PLA2 was found to be followed by the increase of PKC activities in both cell lines. In spite of these similarities, J774 cells possessed both basic and acidic forms of PKC activities, while P 388D1 cells owned only PKC of basic form. Nevertheless, the question why J774 cells, but not P388D1 cells, can acquire the tumoricidal activity, aganist P815 cells following LPS−treatment remains to be answered.

Key Words: *murine macrophages, LPS−induced activation, PLC, PLA_2, PKC.*

INTRODUCTION

Murine macroph, ges and macrophage−like cell lines acquire the abilities to kill tumor cells and bacteria following the interaction with lymphokines such as interferon−γ (INF−γ), and/or bacterial cell wall components, such as lipopolysaccharide (LPS) [1—2]. LPS may initially activate phosphatidylinositol−4, 5−bisphosphate (PIP_2)−specific phospholipase C (PLC) and lead to the development of the tumoricidal activity of macrophages [3]. Jakway and DeFranco [4] proposed that LPS may activate inhibitory guanine nucleotide−binding (Gi) protein, which may be coupled to adenylate cyclase (AC) of a macrophage−like cell line, $P388D_1$. Macrophages respond to LPS also by increased synthesis and secre−tion of various immunoregulatory molecules including prostaglandins (PGs)[4], tumor necrosis factor [5], or IL-1 [6], suggesting a possible activation of phospholipase A_2 (PLA_2) by LPS as well, which may also involve the participation of another type of G protein [7]. Diacylglyceride (DAG), one of the second messengers produced as a result of PLC activation, could be cleaved by DAG-specific lipase to liberate a precursor of PGs [8]. Thus, the initial target of LPS action could be either PLC, PLA_2, or AC, all of which have been shown to involve or require the participation of various types of G proteins.

The studies described above have provided evidence that the activation of PLA2 and PLC was involved in LPS induced early transmembrane signal transduction. Since LPS-mediated DAG enhancement may lead to activation of PKC (9), in turn the activation of PKC may play an important role in LPS−triggered signal transduction pathways (10). In order to examine the possible activation of PKC, the PKC activity of J774 and P388D1 cells were deter−mined.

MATERIALS AND MATHODS

Cells

The murine 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 for macrophages [11, 12]. P388D_1 cells were grown as suspension in a spinner flask at 37℃ in an atmosphere containing 5% CO_2 in culture medium consisting of RPMI 1640, heat-inactivated (56℃, 30min) fetal calf serum (FCS, 10%), streptomycin (100 μg/ml), penicillin (100 units/ml) (All from Hazelton Dutchland, Denver, PA). J774 cells were grown in a basically identical manner to P388D_1 cells, except that Dulbecco, s modified Eagle's (DME) medium was used in place of RPMI 1640 medium. Cell density was maintained between approximately 5×10^5 and 1×10^6/ml. Under these culture conditions, the generation time of two cell lines was about 24hr.

The cell line, P815 mastocyloma from a DBA/2 mouse, was obtained from the American Type Culture (Rockeville, MD) and was cultured in DME medium, containing 10% FCS in a similar manner to J774 cells.

Radiolabeling with [9, 10-^{3}H] Oleic acid

P388D_1 or J774 cells ($2—4\times10^7$) were incubated at 37℃ for 1hr with 50—100 μCi of [9, 10-^{3}H] oleic acid (15Ci/mM, American Radiolabeted Chemicals, St. Louis, MO) in 2.5—5.0ml of Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS, Flow Lab., Rockeville, MD) containing 10 m*M* LiCl and 10m*M* Hepes (HBSS−LH) [9, 13]. The labeled cells were washed three times with HBSS−LH before the stimulation with LPS (from E. Coli 0111:B4, List Biologicals Lab., Campbell, CA). This labeling procedure generally gave the radioactivity of 0.5—1.0 cpm/cell The analysis of chloroform/methanol (2/1, v/v) extract of the radiolabeled P388D_1 cells by thin layer chromatography carried out as described below showed that 52% of the radioactivity was associated with phosphatidylethanolamine (PE), 13% with phosphatidylcholine (PC), 27% with the combined fraction of phosphatidylserine (PS)and phosphatidylinositol (PI), and 9% with the free fatty acid and neutral lipid fraction. The analysis of the lipid extract of J774 cells showed that 65% of the radioactivity was associated with PE, 23% with PC, 4% with PS/PI, and 8% with fatty acid and neutral lipids.

Stimulation of cells

The cells metabolically radiolabeled with [9, 10^{-3}H]oleate were aliquoted into a Corex tube (17 x 100mm) (0.8×10^6 cells/tube)iu 0.4ml of HBSS-LH and stimulated for varying periods of time at 37℃ with LPS (2 to 20 μg/ml). After each incubation period, the cells were treated at 0℃ for lhr with cold 0.2% Triton-X100 (5ml). The detergent lysate was centrifuged for 1 hr at 4℃ and at 15,000× g. The lipide were extracted from the resultant supernatant with 5ml of cold chloroform/methanol (2/1, v/v). The extracted lipids (1.5ml) were dried under a stream of N_2, redissolved in 200 μl of chloroform, and subjected to ascending thin layer chromatography (TLC) as described below.

Fractionation of lipid extracts

Separation of 1, 2−DAG and oleic acid was carried out by asending TLC on a silica gel-coated glass plate (Sil G-25, Sybron/Brinkman, New York, NY.), which was previously heat−activated (1hr, 110℃), as described by Skipski and Barclay [14]. Chloroform/methanol−extracts were first mixed with about 0.1 μg each of authentic nonradioactive 1, 2−DAG (Rf=0.34) aad oleate (Rf=0.57) (both from Sigma, St. Louis, MO) as internal standards. After spotting samples, the chromatography was first carried out for 20min with the use of solvent system consisting of isopropylether/acetic acid (96/4, v/v). aud then for additional 20min with the use of solvent system consisting of petroleum ether/diethylether/aceticacid (90/10/1, v/v/v). Lipid fractions were identified by the exposure of a TLC plate to iodine vapor, scraped from the plate, transferred into scintillation vials, and processed for counting in a β counter. The results were expressed as the percentage of control, based on the values obtained from triplicated assays. Each experiment was repeated three times.

Fractionation of various phospholipids was carried out by ascending TLC of lipid extract on a silica gel G−25 coated glass plate with solvent system consisting of chloroform/methanol/ammonium hydroxide (13/7/1/, v/v/v) [15].

IP_3 assay

Quantilative analysis of inositol−1, 4, 5−triphosphate (IP_3) was carried out with the use of [^{3}H] IP_3 assay system (Amersham, Arlington Heights, IL) according to the manufacturer's method as follows. The cells (4×10^6/tube)were incubated for varying period of time at 37℃ with or without LPS (2—20 μg/ml) in 0.4ml of HBSS−LH[9, 13]. At the end of the incubation period, each tube was added to 0.2ml of trichloroactetie acid (TCA, 15%, w/v) and kept at 0℃ for 20min. The tubes were then centrifuged for 15 min at 2,000×g and at 4℃. IP_3 in the resultant supernatant was extracted three times with 10 volumes of water-saturated diethylether. The extract was neutralized by addition of sodium bicarbonate to pH7.5. The amount of IP_3 in the extract was estimated in duplicate by comparison to a standard curve.

Preactivation of cholera and pertussis toxins

Toxins (10 μg/ml) (Sigma) in 10m*M* Tris-HCl buffer (pH 7.5) containing 10mM dithiothreitol (DTT) and 1m*M* ATP were incubated for 15 min at 30℃[16].

^{51}Cr- labeling of P815 cells

P815 cells were radiolabeled for 1 hr at 37℃ with 200 μCi of ^{51}Cr per 1×10^7 cells by using ^{51}Cr-labeled sodium chromate (specific activity of 300 to 500 mCi/mg, ICN Biochemicals, CA). ^{51}Cr-labeled P815 cells were washed once, centrifuged, and were allowed to leak for 1 hr at 37℃ in complete DME medium with 10% FCS [18].

Macrophage cytotoxicity assay

Killing of radiolabeled target cells (P815 mastocytoma cells) was assayed by using a ^{51}Cr-release assay [18]. Briefly, $P388D_1$ or J744 cells (1×10^5 cells/well) were plated in a well of 96 flat bottom well tissue culture plate and were cultured for 2 hr at 37℃ with 0.2ml of RPMI 1640 medium containing 10% FCS with or without LPS (10ng/ml or 10 μg/ml).Supernatants were then aspirated, and monolayers were washed twice with RPMI 1640 medium. ^{51}Cr-labelled target cells (2×10^4/well) were added to each well in 0.2 ml RPMI 1640 medium containing 10% FCS (Effector:Target ratio of 10:1). Each treament was assayed in triplicate. Each experiment was repeated at least three times. Following the co−culture of 16 hr, the aliquot (0.1 ml) of supernatants was removed from each well and was assayed for the radioactivity in a gamma counter. Results were expressed as percent specific ^{51}Cr release, as calculated by the following equation:

% Specific ^{51}Cr release=100× (experimental release-spontaneous release) / (total releasable counts-spontaneous release)

Spontaneous release was measured in wells where untreated macrophages were incubated with ^{51}Cr-labeled taget 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 purification of PKC

This technique was carried out by using some modifications of the method of Fan et al [17]. Briefly, J774 or P388D1 cells (2×10^7/group) were cultured for 3 hr with or without LPS (1 μg/ml) in RPMI 1640 medium containing FCS, penicillin and streptomycin. At the end of incubation period, the cells were washed with ice−cold Buffer A (5m*M* Tris-HCl, pH7.5, 5m*M* NaCl, 0.5m*M* $MgCl_2$, lm*M* dithiothreitol (DTT), 10 μg /ml of leupeptin), resuspended in 4ml of Buffer A, and homogenized. The homogenates were centrifuged for 1 hr at 100,000×g at 4℃

With the use of HPLC to a column (7.5 ×7.5mm) of DEAE-5PW (Beckman) previously equilibrated against Buffer B (20m*M* Tris-HCl, pH 7.5, containing 0.5m*M* EDTA, 0.5m*M* EGTA, 2m*M* DTT). proteins were first eluted at 1ml/min with 30ml of Buffer B and then with additional 30ml of Buffer B containing 50m*M* NaCl and a total of 60 fractions containing lml each was collected. PKC activity was assayed within 24hr as follows.

PKC assay

An aliquot (50 μl) of each fraction from DEAE−5PW column chromatographp was mixed with 50 μl of PKC assay cocktail (20 m*M* Hepes/NaOH buffer, pH7.5, containing 10 m*M* $MgCl_2$, 3m*M* Ca^{2+}, 120 μg of phosphatidylserine (PS), 12 μg of 1, 2-diacylglycerol and 1mg of lysine-rich histone III per ml and lm*M* DTT, all reagents from Sigma). The basal activity was measured in the presence of 2m*M* EGTA instead of

PS, DAG and Ca^{2+}. Reactions were initiated by addition of 10 μl(0.25uCi) of(γ −^{32}P) ATP(Specific activity, 6000Ci−/mMol, NEN, Boston. MA) and carried out for 20min at 30℃. The ^{32}P-labeled histone III was isolated by pipetting 20 μl of the reaction mixture onto Whatman P-81 phosphocellulose paper (1.5cm× 1.5cm). The papers were then washed with 10% of trichloroacetic acid (TCA) and dried, and counted with a beta counter. The measurement of protein was carried out using the protein BCA protein assay reagents (Pierce, Rockford, IL).

RESULTS

Tumoricidal activities of J774 and P388D$_1$ cells

J774 and P388D$_1$ cells share typical macrophage characteristics such as the adherence to glass or plastic surface, the expression of Fc γ , receptors on their surface, the phagocytosis of opsonized particles or the production of IL-I. However, they were derived from different tumor cells originated in different mice and thus may differ in some other functions of macrophages. Comparative biochemical analysis of those particular functions may provide valuable information on the regulatory mechanisms of macrophage functions. We have therefore first compared the LPS−induced cytotoxicity of P388D$_1$ and J774 cells, using TNF−insensitive P815 cells as target, as described in the Methods.

The results showed that in three separate experiments, the treatment of J774 cells wish LPS at either 10ng/ml or 10 μg/ml invariably activated these cells for specific killing of 30 to 64% of P815 cells. On the other hand, the treatment of P388D$_1$ cells with LPS in two separate experiments failed to develop tumoricidal activity against P815 cells.

Effects of LPS on PLC and PLA$_2$

Since LPS has been shown to regulate the activities of PIP$_2$−specific PLC [4] and/or PLA$_2$ [5], the question of whether or not the above results could be a reflection of the differences in the responses of PLC or PLA$_2$ to exogenously−added LPS was investigated. P388D$_1$ or J774 cells which were previously biosynthetically radiolabeled wish [9, 10-^{3}H] oleate were cultured wish or without LPS (2 μg/ml) for 0 to 40 min. At the end of each incubation time, the cells were lysed with

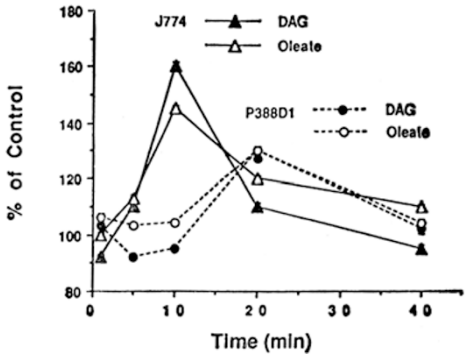

Fig. 1 Time course of LPS−stimulated release of 1,2−[^{3}H] DAG and [^{3}H] oleate from J774 and P388D$_1$ cells. J774 and P388D$_1$ cells which were separately metabolically radiolabeled with [9, I0−^{3}H] oleate were cultured separately for 0 to 40 min with LPS (2 μg/ml). At the end of each incubation period, lipids were extracted and analyzed by TLC as described in the Materials and Methods. The radio−activities associated with [^{3}H]DAG and [^{3}H]oleate extracted from J774 cells at time 0 were 2,054±410 and 6,803±410, respectively, whereas those from P388D$_1$ cells at time 0 were 384±45 and 949±66, respectively. Over the course of experiment, the release of both [^{3}H] DAG and [^{3}H] oleate from LPS−unstimulated controls did not rise above 20% of initial values. Data are representative of 3 similar experiments.

Triton X−100 treatment. Lipids extracted from the detergent lysate were mixed with internal standards, nonradioactive l, 2−DAG and oleate, and subjected to the two−step TLC, as described in the Materials and Methods. The DAG and oleate fractions were identified by the exposure to iodine vapor, scraped from a TLC plate, and counted in a β counter. Results showed, as illustrated by Fig. 1, that both cell lines responded to LPS by an increased release of both [^{3}H] DAG and [^{3}H] oleate, suggesting simultaneous activation of PLC and PLA_2 by LPS. However, the responses of J774 cells wore maximum at 10 min after the addition of LPS, whereas those of $P388D_1$ cells took 20 min io reach the maximum. Based on these results, the treatment periods of 10 and 20 min were used in subsequent experiments with J774 and $P388D_1$ cells, respectively.

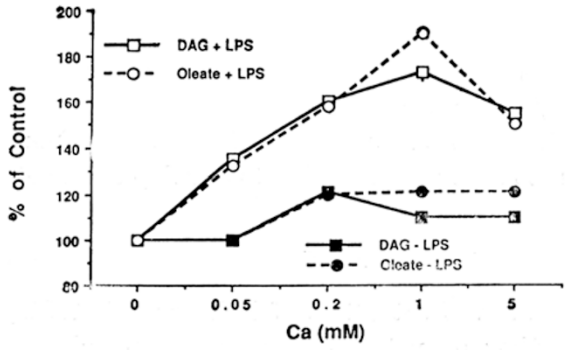

Fig. 2 The effects of calcium on the basal and LPS-stimulated release of [^{3}H] DAG and [^{3}H] oleate from J774 cells. The cells were incubated with or without LPS(2 μg/ml) in the presence of various concentration of Ca^{2+}. The radioactivities associated with DAG and oleate extracted from J774 cells incubated in the absence of Ca^{2+} and LPS (control) were 866±97 and 3, 356± 506, respectively, whereas those in the absence of Ca^{2+} and in the presence of LPS (2 μg/ml) were 1,169±106 and 4,362d±608, respectively. Data are representative of 3 similar experiments.

Effects of Ca^{2+} on the LPS-induced activation of PLC and PLA_2

Previous studies by numerous laboratories demonstrated that the activation of PLA_2 depends on the presence of millimolar Ca^{2+}, whereas that of PLC generally requires micromolar or less Ca^{2+} [19]. As shown by Fig. 2, the addition of Ca^{2+} to the incubation medium significantly increased LPS-induced release of both [^{3}H] DAG and [^{3}H] oleate from J774 cells in a dose-related manner up to the concentration of 1 m*M*. A similar result was also obtained with $P388D_1$ cells, although the magnitude of the effects of Ca^{2+} was less dramatic .(data not shown).

Effects of GTP: γ S on the LPS−induced activation of PLC and PLA_2

Guanosine 5−[γ −thio] triphosphate (GTP γ S), an unhydrolyzable analog of GTP, has been shown in many systems to bind and activate G proteins [20—24] The addition of GTP γ S in the incubation medium increased, in a dose−dependent manner within a concentration range between 0 and 1m*M*, the release of both

[^{3}H] DAG and [^{3}H] oleate from LPS−stimulated P388D$_1$ cells(data not shown). As shown by Fig. 3, the addition of GTPγS(1m*M*) to the incubation medium increased the release of bosh [^{3}H] DAG and [^{3}H] oleate from LPS−untreated J774 cells to an approximately same extent as that from LPS−treated cells. The presence of GTPγS further significantly increased the LPS−induced release of both [^{3}H] oleate and [^{3}H] DAG to 140 and 160% of the control level, respectively. These data thus suggested that the LPS−induced activation of both PLC and PLA$_2$ in two different cell lines may involve the participation of G proteins.

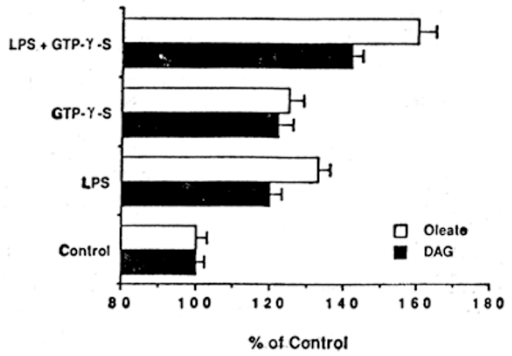

Fig. 3 The effects of GTPγS on the basal and LPS-stimulated release of [^{3}H] DAG and [^{3}H] oleate from J774 cells. The cells were incubated with or without LPS(2 μg/ml) in the presence or absence of GTPγS (1 mM). The radioactivities associated with DAG and oleate obtained with J774 cells in the absence of GTPγS and LPS(control) were 749±42 and8,968±209, resepectively data are representative of 3 similar experiments.

Effects of cholera and pertussis toxins

Effects of cholera and pertussis toxins were investigated because of their known capacities to modulate, through ADP−ribosylation, functions of certain G proteins which couple receptors to effector molecules[7, 25—29]. As shown by Fig 4, the 2 hr−preincubation of J774 cells with cholera toxin at 5μg/ml reduced the basal releases of both [^{3}H] DAG and [^{3}H] oleate to about 50 and 60% of the control level, respectively. The LPS−stimulated release of [^{3}H] DAG and [^{3}H] oleate from J774 cells (140 and 135% of the control level, respectively) was also reduced by CT pretreatment to about 110 and 85% of the control level, respectively. The results with P388D$_1$ cells were similar to those obtained with J774 cells(data not shown). The noted effect of cholera toxin was not apparent when the preincubation time was shortened to less than 1 hr. Pertussis toxin at 100ng/ml, when incubated with J774 or P388D$_1$ cells for 2hr, also reduced the

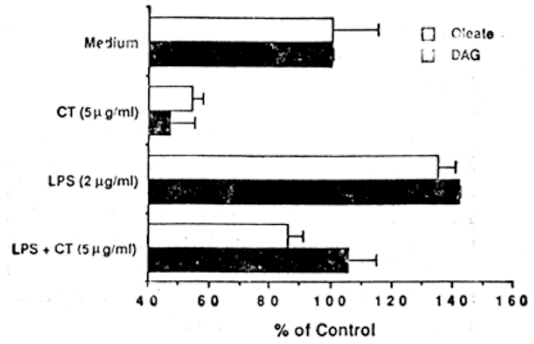

Fig. 4 The effects of cholera toxin (5μg/ml) on the basal and LPS−stimulated release of [^{3}H] DAG and [^{3}H] oleate from J774 cells. The cells were preincubated for 2hr with activated CT and then stimulated with LPS(2μg/ml). The radioactivities (cpm) associated with DAG and oleate extracted from the cells incubated without CT and LPS were 1,938±58 and 2,371±261, respectively. Data are representative of 4 similar experiments.

LPS−stimulated release of both [^{3}H] DAG and [^{3}H] oleate to the basal level (Data not shown). These data thus supported the notion that LPS−induced activation of PLC and PLA_2 involves G proteins which are sensitive to cholera and pertussis toxins.

Effects of enzyme inhibitors on LPS−stimulated release of DAG and oleate

Next, the question of whether or not LPS−stimulated release of [^{3}H] DAG and [^{3}H] oleate is indeed a consequence of the activation of PLC and PLA_2, respectively, was investigated with the use of enzyme inhibitors, as follows. As shown by Fig. 5, the addition of neomycin (NM, 100μM), a PLC inhibitor [20, 30—31], in the culture medium reduced the basal or LPS−stimulated release of [^{3}H] DAG from J774 cells to about 80 and 100% of the control, respectively. The presence of NM increased the basal release of [^{3}H] oleate to 140% of the control and further augmented the LPS−stimulated release of [^{3}H] oleate from 140% to 180% of the control. The effects of neomycin at 10μM were similar to those at 100 μM, to a less profound degree. The addition of p−bromophenacylbromide (pBPB, 10 μM), a PLA_2 inhibitor [32], in the culture medium did not affect the basal release of [^{3}H] oleate or [^{3}H] DAG from J774 cells, but reduced the LPS-stimulated releases of [^{3}H] oleate and [^{3}H] DAG from 140 and 135% of the control to about 90 and 120% of the control respectively. Simultaneous treatment of J774 cells with NM (100μM) and pBPB (10μM) totally blocked LPS−

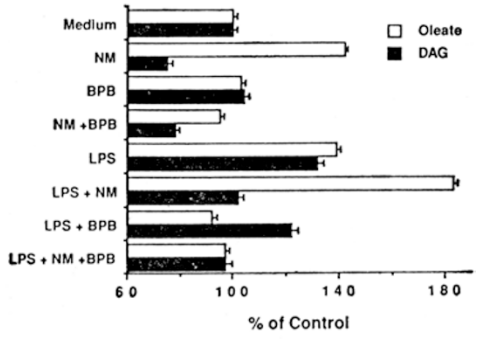

Fig. 5 The effects of inhibitors of PLC (100μM neomycin) and PLA_2 (10μM pBPB) on the basal and LPS−stimulated release of [^{3}H] DAG and [^{3}H] oleate from J774 cells. The cells were with or without LPS(2μg/ml)in the presence of NM, pBPB, or both inhibitors. The radioactivities (cpm) associated with DAG and oleate extracted from the cells incubated in the medium alone were 1,570±100 and 2,289±214, respectively. Data are representative of 3 similar experiments.

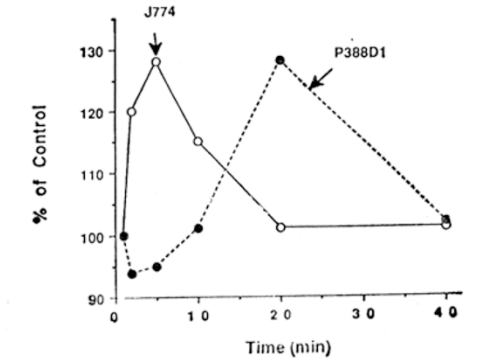

Fig. 6 The formation of IP_3 by J774 and $P388D_1$ cells in response to LPS. J774 or $P388D_1$ cells were incubated with or without LPS(2μg/ml)for 0 to 40min. At the end of each specified incubation period, IP_3 levels were assayed with the use of IP_3 assay kit as described in the Materials and Methods. The radioactivities (cpm) of IP_3 in LPS−unstimulated J774 or $P388D_1$ cells at time 0 were 1756±156 and 2140±106, respectively. IPs levels in unstimulated controls did not rise above 20% of initial values. Data are representative of 3 similar experiments.

stimulated release of both [^{3}H] DAG and [^{3}H] oleate. The results with P388D$_1$ cells were similar to those with J774 cells (data not shown). Thus, the inhibition of LPS−induced or basal PLC activities by neomycin appears to activate both basal and LPS−stimulated PLA$_2$ activities. The inhibition of PLA$_2$ activity with pBPB appeared, on the other hand, not to significantly affect the basal as well as LPS−stimulated PLC activities.

In order to further confirm the above results, the effects of dexamethasone (DEX), another PLA$_2$ inhibitor [33], were also examined. When cells were incubated with 0.05 to 0.5m*M* DEX for 2hr prior to LPS stimulation, the release of both [^{3}H] DAG and [^{3}H] oleate from either cell line was reduced to the control level. DEX did not appear to affect the basal release of either lipids (data not shown).

LP$_3$ response

The above results suggesbed that LPS stimulation of J774 orP388D$_1$ cells leads to the simultaneous activation of PLC and PLA$_2$, both of which may be regulated through cholera and pertussis toxin−sensitive G proteins. To examine whether LPS stimulation actually activates PIP$_2$−specific PLC, the formation of IP$_3$ in response to exogenously−added LPS was also investigated with the use of the IP$_3$ assay kit. As shown by Fig. 6, J774 cells responded to LPS(2μg/ml) by rapid formation of IP$_3$, which reached maximum in 5 min after the addition of LPS. The response of P388D$_1$ cells was again found to be delayed and reached maximum in 20 min after the addition of LPS.

Effect of LPS on PKC activity

Calcium−and phospholipid−dependent protein kinase C (PKC)plays a key role in transmembrane signaling of a wide variety of extracellular stimuli, including growth factors, hormones, antigens and other biologically active substances (34). Diacylglycerol (DAG) is a product from phosphatidylinositol turnover by PLC and it increases PKC affinity for Ca^{2+}, thus resulting in an activation of PKC. The PKC requires Ca^{2+}, DAG and phospholipid (PS) for full enzymatic activation (34). Since LPS was found to increase DAG production we next sought 1). whether LPS induces enhancement of PKC activity of J774 and P388D1 cells; 2) .whether there are any differences of PKC activities of J774 and P388D1 cells in response to LPS. Quantitative estimation of the levels of PKC of J774 and P388D1 cells was carried out. The PKC assay of cytosol of LPS−unstimulated J774 cells fractionated by HPLC on a column of DEAE−5PW clearly showed the presence of two PKC active fractions (basic peak I between the fraction number 5 and 9 and acidic peak II between the fraction number 38 and 40), whereas that of unstimulated P388D1 cells showed only one peak corresponding to the basic form of J774 cells. The treatment of J774 and P388D1 cells with LPS for 3hr enhanced the basic form kinase activity present in J774 and P388D1 cells about 1.5 to 2.0−fold. The acidic form protein kinase activity present in J774 cells also increased about 1.5-fold upon LPS−treatment. Interestingly, LPS−treatment of P388D1 cells did not generate the acidic form of PKG. Time course studies showed that LPS−induced

enhancement of protein kinase activities in both peak I and peak II of J774 cells and in peak I of P388D1 cells was maximal at 3hr following LPS stimulation (data not shown).

DISCUSION

The LPS−triggered biochemical sequence of events which leads to the development of tumoricidal activation ef macrophages has been the subject of intense studies [1—2], and several probable signal−triggering mechanisms have been suggested. Jakway and DeFranco [4] suggested that pertussis toxin−sensitive Gi proteins may play a critical role in mediating the effects of LPS to P388D1 cells or to a B cell Iine (WEHI−231). Since LPS apparently did not affect PIP_2−hydrolysls in either cell lines in their studies, they postulatod that the effects of LPS may be an inhibition of adenylate cyclase mediated by activation of Gi protein coupled to this enzyme. Prpic et al.[3] presented evidence that the interaction of thioglycollate or peptone−elicited peritoneal macrophages with LPS or lipid A promptly triggers the generation of IP_3. Our data showing the LPS−stimulated release of [^{3}H] DAG or IP_3 from two different macrophage−like cell lines are generally in agreement with the results of Prpic et al. The observed release of [^{3}H] DAG or IP_3 in reponse to LPS−treatment is most likely due to the activation of PLC, because the prior treatment of the cells with a PLC inhibitor, neomycin [20, 30—31], prevented the release of [^{3}H] DAG. Although the release of [^{3}H] DAG could be from any membrane phospholipids that incorporated [^{3}H] oleate, the data of Fig. 6 clearly indicate that LPS indeed activates PIP_2−specitic PLC.

The data of Fig. 1 suggest that LPS simultaneously activates PLC and PLA_2 in both cell lines, because the time course of the LPS−stimulated release of [^{3}H] DAG coincided with that of [^{3}H] oleate. However, the results of the IP_3 assay with J774 cells(Fig. 6) suggested that the activation of PLC in response to LPS may precede that of PLA_2 by about 5 min in these cells. In some cell systems, whore both PLC and PLA_2 are activated through receptors for Ca^{2+}−mobilizing hormones, PLC activation has been shown to be prerequisite for PLA_2 activation [21, 35]. The receptor−mediated activation of PLC could activate PLA_2 by IP_3−mediated mobilization of Ca^{2+} from intracellular storage, by supplying DAG or its metabolite, phosphatidic acid, as substrate, by activation of protein kinase C, and/or by causing Ca^{2+} influx. However, the data of Fig. 5 suggest that the LPS−induced activation of PLC and PLA_2 is probably a parallel event, rather than a sequontial activation, because the inhibition of PLC activity with neomycin did not block LPS-induced PLA_2 activation and the inhibition of PLA_2 activity with pBPB did not significantly affect the LPS−induced PLC activation. Another inhibitor of PLA_2, dexamethasone, was found in this study to inhibit not only LPS-induced activation of PLA_2 but also PLC. The effects of dexamethasone could be mediated by lipocortin, which has been shown to inhibit both PLC and PLA_2 [36]. However, whether lipocortin lndeed inhibits both enzymes in response to

LPS treatment needs to be clarified in future, since De George et al. [35] reported that the treatment of C62B glioma cells with glucocorticolds, which increased lipocortin level by 80% following acetylcholine stimulation, inhibits acetylcholine-stimulated PLA_2, but not PLC.

Numerous previous reports indicated that exogenously-added GTP or GTP γ S stimulate or augument hormone-activated PLC in various cell types [19—24]. These findings thus suggested that the activation of PIP_2-specific PLC by extracellular stimuli, such as vasopressin, epinephrine, thrombin, 5-hydroxytryptamine, thyrotropin-releasing hormone, chemotactic peptide, or LPS, is mediated through a G protein, denoted as Gp [21-24]. The data of Fig. 3 showing the up-regulation of the release of [^{3}H] DAG and [^{3}H] oleate by GTP γ S suggest a probable involvement of G proteins in the LPS-induced activation of both PLC and PLA_2 in J774 or $P388D_1$ cells. PT has been shown to inactivate the function of Gp in some cells as seen in the studies of Smith et el. and Kikuchi et el. [23—24] ,but not in others [21]. This apparent difference could be due to the heterogeneity of Gp as well as PLC among various cell types. Probable coupling of PT-sensitve G protein to the receptors which trigger the arachidonic acid release has been described in mast cells [25], neutrophils [26], fibroblast [27], and FRTL5 thyroid cells [7]. The question remains whether or not a G protein mediating the activation of receptor-triggered activation of PLA_2 is the same as Gp protein, because in several previous reports, as in this study, PT has been shwon to inhibit both PLC and PLA_2, whereas Burch et al. clearly demonstrated that norepinephrine-stimulated PLA_2, but not PLC, is sensitive to PT [7].

In a variety of cells, CT has been reported to inhibit, totally or partially, receptor-stimulated IP_3 release [28—29]. In J774 or $P388D_1$ cells, CT was found to be an effective inhibitor of both basal and LPS-stimulated PLC and PLA_2 (Fig. 4). However, whether or not CT directly affected the functions of Gp proteins in two macrophage-like cell lines needs to be clarified by further studies. The noted effects of CT on PLC and PLA_2 could be a consequence of the activation of Gs protein coupled to the adenylate cyclase system, since the elevation of intracellular cAMP level has been reported to inhibit the PIP_2-turnover in neutrophils [37], lymphocytes [38—39], thymocyles [40], and platelets [41]. However, it is unclear at present if this is indeed the case, because CT treatment of BALB/c/3T3 cells was reported to increase the EGF-and PDGF-stimulated IP_3 accumulation [42].

The data presented in this paper clearly showed that the maximum PLC and PLA_2 response of $P388D_1$ cells to LPS lags at least 10 to 15 min behind that of J774 cells, although both the magnitude and sensitivities to various agents appear to be similar between two cell lines. Neither the causes of this difference nor the relationship of this difference to the difference in the development of tumoricidal activity toward P815 cells is clear at present. However, LPS-induced activation of PLC and PLA_2 is probably an important initial step to develop tumoricidal activity. In our recent preliminary studies, both DEX and CT, which are shown to suppress LPS-indueed activation of these enzymes, are found to effectively block the tumoricidal activation of J774 cells (manuscript in press). The failure of

$P388D_1$ cells to gain tumoricidal activity could thus be in the steps which follow the initial activation of PLC and PLA_2.

The LPS−induced accumulation of DAG and oleate was indicative of activation of both PLA_2 and PLC. The interaction of ligand with receptor results in a transient hydrolysis of cell membrane phosphoinositides that are cleaved by PLC to generate 1, 2−diacylglycerol (DAG) and 1, 4, 5−trisphosphate (IP3), in turn, both directly activate Ca^{2+} signal and Ca^{2+}−dependent PKC[10].

It has been reported that unsaturated fatty acids (oleate and arachidonate) can activate PKC directly [43]. These evidences clearly suggest that the activation of PLA_2 and PLC may switch on the activation of J744 and P388D1 cells in response to LPS.

Protein kinase C (PKC) has been received considerable attention recently due to its apparent central role in cellular regulation and function [10, 44], as well as in activation of monocytes−macrophages [45]. The PLC activaties of J744 and P388D1 are likely to be influenced in response to LPS. In order to examine this bypothesis, the analysis of PKC activaties of J744 and P388Dl cells was undertaken. As our results indicated that PKC activities can be modulated by LPS, since LPS−unstimulated J744 and P388D1 cells only contain about half of PKC activity of LPS−stimulated J744 and P388D1 cells. The cytosol PKC activities of J744 cells appeared in two factions (basic and acidic peaks) separable by DEAE-5PW HPLC, while these P388D1 cell showed only one signal peak (basic form). These results clearly suggested that PKC within J744 cells was heterogeneous.

The molecular heterogeneity of PKC has been reported and seven isoenzymes of PKC have so far been identified [44]. Thus, the charcterization of heterogeneity between J744 and P388D1 cells is being studied. In addition, the evidence that calpain may regulate the activity of PKC has been presented by others [46]. PKC can be cleaved by a neutral protease (calpain) to produce a catalytically active fragment (protein kinase M) [46]. Thus, it is interesting to know whether the noted differences of PKC between J744 and P388D1 cells is due to the heterogeneity of PKC properties or is due to the regulatory and catalytic domain fragments of PKC cleavagd by calpain. Experiments are being conducted.

Further studies of the role of PKC in LPS−induced intermediate and late events, such as, PKC−mediated protein phosphorylation, the regulation of DNA−binding protein as well as proto−oncogene and cytokine gene expression, should help to elucidate the correlation of LPS−triggered transmembrane signaling transduction and tumoricidal properties of macrophages.

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