Isolation of a Nitric Oxide Inhibitor from Mammary Tumor Cells and its Characterization as Phosphatidyl Serine

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Summary

Macrophages from mice bearing large D1-DMBA-3 mammary tumors have a decreased capacity to kill tumor targets. This effect is due to an impaired ability to produce nitric oxide (NO) in response to lipopolysaccharide (LPS) stimulation. Here we report that the DA-3 tumor cell line, derived from the in vivo adenocarcinoma D1-DMBA-3, produces a factor that inhibits both NO production/release and cytotoxicity of LPS-activated peritoneal exudate macrophages (PEM). However, other complex macrophage functions such as phagocytosis, superoxide production, mitochondrial dehydrogenase activity, and synthesis of proteins were not reduced by this factor. The NO inhibitor has been found to be lipid in nature. Lipid extracts from DA-3 cell culture supernatants were purified by repeated silica gel column chromatography. The active molecule was unambiguously characterized as phosphatidyl serine (PS) by fast atom bombardment tandem mass spectrometry. Preliminary results indicate a lack of induced NO synthase (iNOS) activity in the lysates of LPS-activated PEM pretreated with PS. The ubiquity of PS in the inner leaflet of biological membranes and its NO inhibitory property, suggest that this phospholipid may be one of the long elusive molecules responsible for regulating physiological levels of NO in the host and hence preventing cellular dysfunction and/or tissue damage. Furthermore, the possible overexpression and shedding of PS by DA-3 tumor cells may represent a novel mechanism to impair macrophage cytotoxicity, a host function that contributes to the protection against developing neoplasms.

Upon activation with a variety of stimuli, macrophages produce factors that lead to lysis of tumor targets. This host defense mechanism has been shown to be mediated by the participation of one or more molecules, which include TNF-α, oxygen-reactive intermediates, and nitric oxide (NO)[1] (1). We previously have shown that peritoneal exudate macrophages (PEM) from mice bearing large mammary tumors have an impaired capacity to kill xenogeneic, allogeneic, syngeneic, and autologous tumor targets (1–3). Recently we have found that PEM from these tumor bearers produce TNF-α, IL-1, and H_2O_2 at levels comparable or higher to those of PEM from normal mice (3). However, they display a depressed capacity to produce NO, an important cytostatic factor for macrophages (4, 5).

In previous studies, we have found that the DA-3 tumor cells synthesize a variety of factors that modulate the host immune response of tumor-bearing mice. Thus, we sought to investigate whether a tumor-derived product was responsible for the inhibited $N\dot{O}$ production observed. We now describe that supernatants of the DA-3 tumor cell line release a factor that inhibits, in a dose-dependent fashion, NO production and the cytotoxic activity of PEM derived from normal mice. This inhibitory molecule has been characterized by enzymatic degradation and mass spectrometry (MS) analysis as phosphatidyl serine (PS) (6, 7).

Materials and Methods

Mice and Tumor Cells. BALB/c mice are maintained by brother-sister matings in our laboratory at the University of Miami School of Medicine. The DA-3 tumor cell line was derived from the in

[1] *Abbreviations used in this paper:* FAB, fast atom bombardment; GC-MS, gas chromatography-mass spectrometry; iNOS, induced nitric oxide synthase; MTT, (3-[4,5-dimethylthiazol-2-y1]-2,5 diphenyltetrazolium bromide); m/z, mass-to-charge ratio; NADPH, *B*-nicotinamide adenine dinucleotide phosphate, reduced form); N^GMMA, N^G-monomethyl-L-arginine, monacetate; NO, nitric oxide; NO_2^-; nitrite anion; PC, phosphatidyl choline; PEM, periotoneal exudate macrophage; PG, phosphatidyl glycerol; PLC, phospholipase C; PS, phosphatidyl serine; SM, sphingomyelin.

vivo D1-DMBA-3 mammary tumor syngeneic to BALB/c mice (8). The DA-3 cells produce tumors in BALB/c mice and express the same tumor-associated Ags on their surfaces as the parent tumor cells. The cell line is grown in RPMI-1640–supplemented media with 5% FCS, 100 U penicillin, 100 μg/ml streptomycin, 5 × 10^5 M 2-ME, 2 mM L-glutamine, 1% nonessential amino acids, 1% essential amino acids, and 1% sodium pyruvate (all from GIBCO BRL, Gaithersburg, MD), and maintained by serial passage. These cells are free of endotoxin as ascertained by routine assays with Limulus amebocyte lysate (Pyrogent® plus; Whittaker M.A. Bioproducts, Inc., Walkersville, MD). The metastatic 410.4 and nonmetastatic 168 cell lines were derived from a spontaneously arising mammary tumor in a BALB/C fC_3H mouse (9) and cultured in Waymouth medium (GIBCO BRL) supplemented with 10% FCS. The human cell lines, A375 melanoma, squamous cell carcinoma A431, and a normal human epidermal keratinocyte (NHEK), were obtained from American Type Culture Collection (Rockville, MD) and cultured as indicated (10).

Reagents. The culture medium used in the experimental assays was RPMI-1640 supplemented with 100 μg/ml streptomycin, 5 × 10^{-5} M 2-ME, 2mM L-glutamine, 1% nonessential amino acids, 1% essential amino acids, 1% sodium pyruvate (all from GIBCO BRL), and 10% endotoxin-free FCS (Hyclone Laboratories, Logan, UT). LPS (*Escherichia coli* 055:B5) from Difco Laboratories (Detroit, MI) was used as indicated. Bovine brain PS extract (Type III: Folch Fraction III), phosphatidyl glycerol ([PG] from egg yolk lecithin), phosphatidyl choline ([PC] from bovine brain), sphingomyelin ([SM] from chicken egg yolk), trypsin, proteinase K NADPH (β-nicotinamide adenine dinucleotide phosphate reduced form), FAD (flavin adenine dinucleotide), PMA, ferricytochrome c from bovine heart, superoxide dismutase from bovine liver, L-lactic dehydrogenase (type XVII from bovine heart), staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus*, and MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) were purchased from Sigma Chemical Co. (St. Louis, MO). DNase, pepstatin A, chymostatin, aprotinin, PMSF, and DTT (dithiothreitol) were obtained from Boehringer Mannheim (Indianapolis, IN). Synthetic 1-stearoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Molybdenum (VI) oxide (MoO_3), zinc chloride, and resorcinol were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI). HPLC grade chloroform and methanol were purchased from Baxter Diagnostics Inc. (McGraw Park, IL). N^Gmonomethyl-L-arginine monoacetate (N^GMMA) and L-arginine were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). BH_4 ([6R]-5,6,7,8-tetrahydro-L-biopterin dihydrochloride) was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Sodium pyruvate was obtained from JRH Biosciences (Lenexa, KS). Murine r-IFN-γ was purchased from Genzyme Corp. (Cambridge, MA).

Macrophage Cultures. Normal mice were injected with 1 ml i.p. of thioglycollate (Difco Laboratories). On day 4, the PEM were obtained by peritoneal lavage with 10 ml ice-cold RPMI-1640/mice. Peritoneal cells were washed twice and resuspended in supplemented RPMI-1640 media. The adherent population was obtained following the plastic-adherent technique described by Pennline (11). This procedure provides a population consisting of >95% macrophages as determined by staining with Diff-Quick differential stain (Baxter Diagnostics Inc.) and nonspecific esterase staining (Sigma Chemical Co.). Viability of cells was routinely >95% by trypan blue exclusion. PEM were seeded into 96-well, flat-bottom microtiter plates (Costar Corp., Cambridge, MA) at a cell density of 1.5 × 10^5/microtiter well.

Collection and Initial Treatments of DA-3 Cell Supernatants. Cells of the DA-3 line (10^5/ml) were continually cultured with supplemented RPMI-1640 and supernatants were collected 3 d after passage, centrifuged at 400 *g* for 10 min to remove cellular components, passed through a 0.45-μm filter (Millipore Corp., Bedford, MA), and stored at −20°C until use. Aliquots of DA-3 supernatants were heated at 100°C for 20 min, or treated with either trypsin (10 μg/ml), proteinase K (0.5 mg/ml), or DNase (100 U/ml) overnight at 37°C. Filtered DA-3 supernatants were extracted twice with a threefold volume of HPLC-grade chloroform-methanol (2:1, vol/vol). The organic phases were combined and concentrated in vacuo, and then transferred to 5-ml glass vials. Samples were taken to complete dryness under a stream of nitrogen. The film of the crude lipid extract contained in the glass vial was weighted and then dispersed in RPMI-1640 by sonication for 7 min in a bath-type sonicator. The lipid suspension was then sterilized by heating (100°C, 20 min) and then resonicated for 7 min before use in 0.2-ml cultures. Before the biological assay, traces of methanol and chloroform present in the aqueous phase were removed with a rotary evaporator in vacuo. This phase was then filtered with a 0.45-μm filter and subsequently sterilized by heating at 100°C for 20 min before use.

Nitrite (NO_2^-) Determination. PEM from normal mice were preincubated for 24 h in the presence or absence of inhibitors. After this time, the macrophages were washed twice and further incubated for 24 h without or with LPS to elicit the production of NO. In some experiments IFN-γ (500 U/ml) or SEB at 300 μg/ml were used instead of LPS as activators. Nitrite concentration in the cell-free macrophage supernatants served as a reflection of NO production and was measured by the colorimetric Griess reaction as described by Stuehr and Nathan (12). Briefly, 100 μl of sample aliquots were mixed with 100 μl of Griess reagent (1% sulfanilamide; 0.1% *N*-[1-naphthyl]ethyl-enediamine dihydrochloride and 2% H_3PO_4) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate reader (BioWhittaker, Inc., Walkersville, MD). NO_2^- levels were determined using $NaNO_2$ as a standard, and double-distilled H_2O as a blank.

Fractionation of Lipids Extracted from the DA-3 Cell Supernatants and Assessment of their NO Inhibitory Property. After concentration of dryness, the crude lipids were resuspended in chloroform and a preliminary separation was carried out by silica gel (230–400 mesh; Association for Standard Test Methods [ASTM], American Scientific Products [ASP], McGauss Park, IL) column chromatography sequentially fractionated with HPLC grade chloroform, acetone, and methanol (13). 5 ml of crude fractions were collected, dried under nitrogren, and dispersed in RPMI-1640 by sonication. The collected fractions were sterilized and preincubated at a concentration of 130 μg/ml with PEM from normal mice (1.5 × 10^5 macrophages/0.2 ml) for 24 h. After this time, the macrophages were stimulated with LPS (10 μg/ml) for an additional 24 h, and the formation of NO_2^- was measured. The fractions were monitored by TLC (250-μm layer flexible plates for silica gel TLC; Whatman Laboratory Products, Clifton, NJ), developed with chloroform-methanol-acetic acid-water (65:15:10:4, vol/vol), and visualized with either iodine vapors, resorcinol reagent (14), or molybdenum blue reagent (15).

Purification of the Active Lipid Fractions. The active fraction (methanol eluate) was concentrated in vacuo at 40°C, resuspended in chloroform-methanol (95:5), charged on a silica gel column as above, and successively eluted with chloroform-methanol (9:5, 80:20, 0:100) mixtures (16). 5-ml fractions were collected and the separa-

tion of the mixture was monitored by TLC, using as a solvent system chloroform-methanol-acetic acid-water (65:15:10:4, vol/vol). The chromatogram was visualized with molybdenum blue. The collected fractions were stored at −20°C until used in the experimental procedures.

HPLC Analysis. The purified fraction from the second chromatographic separation containing the inhibitory activity (fraction 46), was dried under nitrogen and resuspended in 1 ml of chloroform. This solution (15 μl) was injected into a HPLC system (Dionex Corp., Sunnyvale, CA) using a 3.9 mm × 30 cm μPorasil™ (Waters Chromatography Div., Milford, MA) with a mobile phase of acetonitrile-methanol-85% phosphoric acid (390:5:4.5, vol/vol) flowing at 2.0 ml/min. UV detection was monitored at 205 nm (17).

Digestion of Fraction 46 with Phospholipase C (PLC). The digestion of phospholipids with PLC from *Bacillus cereus* has been described elsewhere (18). Briefly, fraction 46 (5 mg) and SM (3 mg) were mixed with 0.2 M phosphate buffer (pH 7.0, 0.5 ml) containing 0.001 M 2-ME, 0.0004 M zinc chloride, and 1 mg PLC from *B. cereus* (Fluka Chemie AG, Buchs, Switzerland) in the same buffer (0.5 ml). After the mixture was shaken vigorously for 2 h at 37°C, it was extracted three times with diethyl ether (4-ml portions). The ether layer was dried over anhydrous sodium sulfate before it was evaporated in a stream of nitrogen. The resulting lipids were separated by TLC (250-μm layer flexible plates for silica gel TLC; Whatman Laboratory Products) with hexane-diethyl ether (50:50, vol/vol) as solvent system and sprayed with iodine vapors. The bands were scraped from the plates and eluted with methanol. The resulting solution was evaporated with a stream of nitrogen, resuspended in RPMI-1640, and sterilized in the same way as fraction 46.

Transesterification of Fraction 46. A 10-μg portion of fraction 46 was dissolved in a 1-N solution of sodium methoxide in absolute methanol (Aldrich Chemical Co.). After 2 h, the reaction mixture was dried under a stream of nitrogen. The liberated fatty acid methyl esters were then dissolved in 20 μl *N*-methyl-trimethylsilyltrifluoroacetamide (MSTFA; Regis Chemical Co., Morton Grove, IL) to which was added 2 μl of 10% 2,4,6-trimethylpyridine in chloroform to trimethylsilylate-free hydroxyl groups. After 15 min at 60°C, the silylation reaction mixture was cooled and used directly for gas chromatography (GC-MS) analysis.

Mass Spectrometry. Fast atom bombardment (FAB) mass spectra were acquired on a HX-110 double focusing mass spectrometer (Jeol Ltd., Peabody, MA) operating in the negative ion and positive ion mode. For the negative ion mode analysis, samples were prepared by mixing 1 μl of the sample solution (0.5–1 μg/μl) with a matrix solution composed of glycerol/15-crown-5/18-crown-6 (1 + 0.5 + 0.5 μl) containing *N*-octyl nicotinium bromide (1 μl of 0.5 μg/μl methanol) on the probe tip. Glycerol (1–2 μl) was used as matrix for the positive ion mode analysis. After introduction of the probe through the vacuum lock, ions were produced by bombardment with a beam of 6 keV Xe atoms. The accelerating voltage was 10 kV and the resolution was set to 1,000. For collisionally activated dissociation tandem MS (CAD-MS/MS), helium was used as a collision gas in a cell located in the first field-free region. The helium pressure was adjusted to reduce the absorbance of the precursor ion by 50%. A data system (model DA-5000, Jeol) generated linked-scans at a constant B/E ratio. The instrument was scanned from zero to 1,500 mass units in 45 s. Data presented were from a single scan.

GC-MS was performed on a double focusing mass spectrometer (model AX505; Jeol) interfaced to a gas chromatograph (model 5890; Hewlett-Packard, Palo Alto, CA) via a heated inlet. A 15 m × 0.32 mm id capillary column (model DB-1701; J & W Scientific, Folsom, CA) was used for the analysis. The temperature program was initiated at 100°C and held for 2 min. At a rate of 25° per min the temperature was increased to 200°C and then at 5° per min at 260°C. The injector and interface temperature were 260° and 280°, respectively. Electron impact ionization at 30eV was employed.

Macrophage Cytotoxicity Assay. Cytotoxicity was determined by the MTT method (19). Briefly, purified PEM from normal mice (1.5×10^5 cells/well) were first incubated in either medium alone, DA-3 cell supernatants, different concentrations of the active fraction 46, or as indicated in Results. After 24 h, supernatants of macrophage cultures were discarded, and the cells were washed twice with warm RPMI-1640. Experimental cultures were stimulated with 10 μg/ml LPS for an additional 24 h. The cultures were washed with RPMI-1640 before addition and coculture with DA-3 targets (10^4/well) for 18 h. MTT (5 mg/ml) in PBS was added to each well, and the microplates were further incubated at 37°C for 4 h. Supernatants were then discarded and 200 μl of acidified isopropanol (0.04 N HCl in isopropanol) were added to the cultures and mixed thoroughly to dissolve the blue crystals of formazan. Formazan quantification was performed using an automatic plate reader (BioWhittaker, Inc.). The absorbance was measured at 570 nm. Data were expressed as mean absorbance value (OD) of triplicate samples ± standard error of the mean. Percent-specific cytotoxicity (% C) was calculated as follows: 100 − [(OD of effectors + targets) − (OD of effectors)/OD of targets] × 100.

Cell Viability. Monolayers of PEM were preincubated for 24 h in the presence or absence of PS. The cultures were vigorously washed with RPMI-1640 and further incubated at 37°C for 4 h with 200 μl of 5 mg/ml MTT in PBS. Formazan formation was assayed as indicated above.

Superoxide (O_2^-) Assay. Superoxide generation was measured as the superoxide dismutase–inhibitable reduction of ferricytochrome c as described by Pick and Mizel (20). Briefly, macrophage monolayers (1.5×10^5 cells) preincubated or not with PS for 24 h were washed twice with RPMI and then stimulated with LPS (10 μg/ml) and PMA (100 ng/ml) for 24 h. After this time, the cell cultures were washed with warm HBSS without phenol red to completely remove the red coloration of the RPMI. Macrophage monolayers were incubated for 1 h with 100 μl of reaction solution containing 160 μM ferricytochrome c in the HBSS without phenol red at pH 7.4. The amounts of O_2^- production per well was calculated from the following formula: nmol O_2^- per well = (absorbance at 550 nm/6.3) × 100. The specificity of the reaction was checked by the addition of superoxide dismutase (300 U/ml) which inhibited at least 90% of the stimulation. The absorbance at 550 nm was measured in a microplate reader (BioWhittaker, Inc.).

Phagocytosis. Coverglass slips were placed on the bottom of 17-mm plastic wells (24-well cell culture cluster, Costar Corp.) to which 3×10^5 PEM from normal mice in 1 ml RPMI were added per well and incubated at 37°C for adherence. Monolayers of adherent macrophages were cultured for 24 h with medium alone or medium with PS (160 μg/ml). The macrophage cultures were washed twice with RPMI and further incubated for 30 min with 3×10^7 zymosan particles (Sigma Chemical Co.) in 1 ml culture medium supplemented with 10 μg/ml LPS. After this period of time, cell monolayers were washed, fixed with 2% paraformaldehyde, and permeabilized with methanol. The coverslips were mounted on a glass slide and the number of zymosan particles phagocytosed by PEM were determined using an oil immersion objective. At least 100 cells were counted to determine a mean number of phagocytosed particles.

Laser Scanning Confocal Microscopy. Experimental PEM were also

viewed on an inverted Nikon microscope which is equipped with a laser scanning device (MultiProbe 2001; Molecular Dynamics, Sunnyvale, CA) using the 488-nm wavelength of the argon/krypton emission lines. The confocal laser scanning images were collected and recorded at 0.6-μm increments. The image processing was performed on a computer (Silicon Graphics, Mountain View, CA), and grey or pseudo colorizations were overlayed on the transmitted light image and the refracted light image respectively. Black and white pictures were taken by a 35-mm camera (Screen Star, Sunnyvale, CA).

Protein Determination. Control and PS pretreated macrophage monolayers were washed twice with RPMI and then incubated at 37°C for 24 h with culture medium plus LPS (10 μg/ml). After removal of the culture supernatants, adherent cells were washed twice and lysed with 0.5 N NaOH. Protein concentration in the lysates was determined by the method of Lowry (21) using a protein assay kit (Sigma Chemical Co.). Optical absorbance was measured at 570 nm.

Measurements of IL-1α and IL-6. PEM experimental culture supernatants from triplicate wells were pooled and IL-1α protein concentrations were measured quantitatively by ELISA (Inter Test-1αX Mouse IL-1-α ELISA kit; Genzyme Corp.). Similarly, IL-6 was measured using a murine IL-6 ELISA kit (Endogen Inc., Boston, MA).

Kinetics of the Inhibitory Activity of PS on LPS-activated PEM. Macrophages were preincubated with 160 μg/ml PS at 37°C for time periods of 2, 4, 6, 8, and 18 h. Subsequently, the cells were washed twice with RPMI and further incubated for 18 h with culture medium containing 10 μg/ml LPS. NO_2^- concentration in the cell-free macrophage supernatants was measured as described above. Another experimental group consisted of macrophage cultures cocultured for 18 h with 10 μg/ml LPS and 160 μg/ml PS. The effect of PS on the nitrite production of LPS-activated macrophages was assayed by adding 160 μg/ml PS to LPS-activated macrophages 2, 4, and 6 h after activation. PS untreated macrophages were used as controls for each experimental group.

Reversibility of the Inhibitory Activity of PS on the Production of NO by PEM. Monolayers of PEM were preincubated at 37°C with medium alone or medium supplemented with 160 μg/ml PS for a period of 24 h. After removal of PS, macrophage monolayers were washed twice with RPMI, fresh medium was added, and activation followed immediately thereafter (zero time) with 10 μg/ml LPS for 18 h or after 24 or 48 h of incubation at 37°C. Macrophage supernatants were analyzed for their content of NO_2^-.

Preparation of Macrophage Cytosol and Measurement of Inducible NO Synthase (iNOS) Activity. This experiment was carried out as described by Bogdan et al. (22). LPS-activated PEM untreated (control) or PS pretreated were resuspended in culture medium and pelleted by centrifugation at 4°C, and resuspended again in 1X PBS and counted in trypan blue. The cells (8.25×10^7) were then repelleted and lysed by three cycles of freezing and thawing in 1 ml of lysis buffer (40 mM Tris/HCl, pH 8, 5 μg/ml pepstatin A, 1 μg/ml chymostatin, 5 μg/ml aprotinin, and 100 μM PMSF). The broken-cell suspension was centrifuged at 18,000 *g* for 30 min. The iNOS activity in the supernatants was measured as L-arginine- and NADPH-dependent generation of NO_2^-. The assay was performed in 96-well microplates (Costar Corp.) by incubation of 200 μg of cytosol at 37°C for 18 h in 200 μl reaction buffer containing 20 mM Tris/HCl (pH 8), 4 μM BH_4, 4 μM FAD, 5 mM DTT, 2 mM NADPH, and protease inhibitors listed above. The reaction was initiated by adding 2 mM of L-arginine. Controls not receiving L-arginine were run in each case. In some experiments, different concentrations of PS or lysates from LPS-activated PEM pretreated with PS were preincubated with lysates of untreated LPS-activated macrophages for 30 min before the addition of L-arginine. The reactions were terminated by addition of 2 U lactic dehydrogenase and 5 μmol pyruvate to oxidate residual NADPH, which interferes with the Griess reaction. The final incubation mixtures were filtered by the use of the Ultrafree-MC Filter-30,000 NMWL (Millipore Corp.) and the filtrates were mixed with the Griess reagent (1:1, vol/vol) for NO_2^- determination. NO_2^- in the control incubation (without L-arginine) was substracted from experimental values to determine L-arginine/NADPH-specific activity. NOS activity in macrophage cytosol was normalized according to the protein concentration of each experiment and expressed as nM of nitrites.

Results

Inhibitory Effect of DA-3 Cell Supernatants on the Production/Release of NO_2^- by PEM. Macrophages from normal mice were pretreated for 24 h with different concentrations of supernatants from DA-3 cell cultures. The macrophage monolayers were then washed twice and stimulated with 10 μg/ml of LPS for an additional 24 h. After this time period, the macrophage culture supernatants were subjected to analysis of NO_2^-, a stable and nonenzymatic oxidative degradation product of NO (23). Fig. 1 shows that pretreatment of normal macrophages with DA-3 cell supernatants caused a dose-dependent inhibition of their production of NO_2^-. 50% DA-3 cell supernatant in the 0.2-ml macrophage cultures (1.5×10^5 cells) appeared to be the concentration necessary to achieve almost complete inhibition of LPS-induced NO. Downregulation of NO production/release by PEM was not attributable to a toxic effect of the DA-3 cell supernatants on the macrophages. Thus, the treated PEM released normal

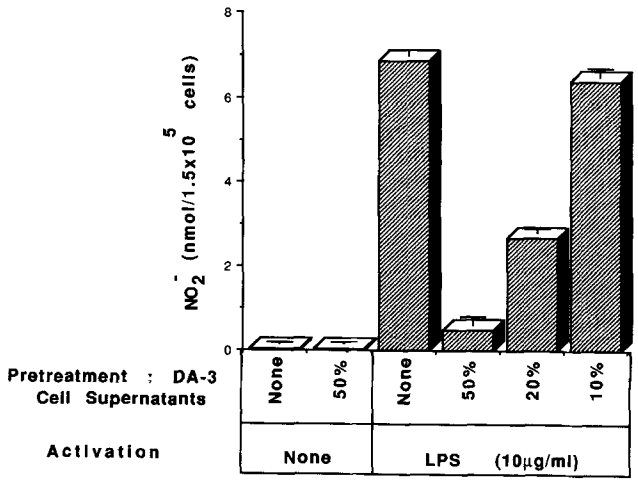

Figure 1. Inhibitory effect of DA-3 cell supernatants on normal PEM production of NO_2^-. PEM from normal mice (1.5×10^5/well) were incubated for 24 h with different concentrations of DA-3 supernatants or RPMI medium. Fresh medium with LPS (10 μg/ml) was added to macrophage cultures for an additional 24 h. The supernatants were collected and NO_2^- was measured as described in Materials and Methods. Values represent mean ± SE of triplicate wells from at least four separate experiments.

levels of IL-1 or IL-6 and their cell viability was not reduced as measured by trypan blue exclusion.

Initial Partial Characterization of the NO Inhibitory Factor(s) Present in the DA-3 Cell Supernatants. As seen in Table 1, treatment of DA-3 cell supernatants at 37°C overnight or boiling at 100°C for 20 min did not impair its NO_2^- inhibitory property. In addition, exposure of the tumor cell supernatants to trypsin (10 mg/ml) or proteinase (0.5 mg/ml) did not diminish the observed inhibition of nitrite production by PEM. Likewise, DNase treatment did not disrupt the bioactivity of the DA-3 cell cultures. These results suggest that the inhibitory factor(s) is a nonproteic molecule(s), most probably lipid or carbohydrate in nature. Therefore, it is unlikely that the inhibitory molecule is one of the previously reported macrophage NO-inhibitory factors of proteic nature, such as macrophage deactivating factor, TGF-β_1, β_2, and β_3 which have been purified from P815 mastocytoma cells (24). Likewise, other NO inhibitors of proteic nature such as epidermal growth factor (25), IL-4 and IL-10 (26, 27) and could not be the bioactive compound. Indeed we have found that the mammary tumor used in our studies does not produce either IL-4 or IL-10 (data not shown). We then performed a chloroform-methanol extraction to investigate whether a lipidlike molecule was involved in the NO inhibition observed. Both the chloroform-methanol–extracted materials and the aqueous phase were tested as potential inhibitors of nitrite production by macrophages. Table 1 shows that 40 μl of the aqueous phase of the chloroform-methanol extraction from DA-3 supernatants had no inhibitory activity on the macrophages production of NO_2^-. Concentrations of the nonlipid aqueous phase >40 μl (20%) were toxic to the macrophages. In sharp contrast were the results obtained when the chloroform-methanol–extracted fraction was used as inhibitor. A slight decrease in NO production was observed with 0.19 mg/ml extract. Pretreatment with 0.75 mg/ml of the lipid extract reduced the levels of NO produced by normal PEM after LPS stimulation to one half of the levels observed in the untreated macrophage cultures. It is important to note that when the chloroform-methanol extract was used at a concentration of 1 mg/ml, this extract displayed a bioactivity comparable to that of 100 μl of the untreated DA-3 supernatant. Since the NO-inhibitory molecule(s) can be recovered in the organic phase after extraction of the DA-3 cell supernatants with chloroform-methanol mixtures, the possible extraction of the highly polar peptide platelet-derived growth factor, a reported inhibitor of macrophage NO production (28), could be excluded.

Table 1. *Preliminary Characterization of the NO-Inhibitory Molecule(s) Produced by the DA-3 Mammary Tumor Cells*

Tumor supernatant treatment	NO_2^- (nmol/1.5 × 10^5 macrophages)
No supernatant	8.20 ± 0.50
Untreated	0.60 ± 0.50
Treated at 37°C overnight	0.58 ± 0.40
Heated at100°C (20 min)	0.51 ± 0.38
Trypsin (10 mg/ml)	0.55 ± 0.50
Proteinase K (0.5 mg/ml)	0.60 ± 0.50
DNase (100 U/ml)	0.60 ± 0.45
Chloroform/methanol extraction (2:1 vol/vol)	
0.19 mg/ml	6.98 ± 0.50
0.75 mg/ml	3.10 ± 0.48
1.0 mg/ml	0.48 ± 0.50
Filtered (0.45 μm) aqueous phase from chloroform/methanol extraction (20%)	8.15 ± 0.50

PEM from normal mice (1.5 × 10^5 cells/0.2 ml cultures) were incubated for 24 h with medium (no tumor supernatants), untreated DA-3 supernatants (50%), or supernatants treated as described above. LPS (10 μg/ml) was added to macrophage cultures for an additional 24 h. The supernatants from the macrophage cultures were collected, and NO_2^- was measured as described in Materials and Methods. Values represent mean ± SE of triplicate wells from a representative experiment.

It should be emphasized that the viability of macrophages under these experimental conditions was not reduced, based upon trypan blue exclusion, morphological evaluation, and IL-1 and IL-6 production.

Fractionation of the Organic Extract and Partial Characterization of the NO Inhibitory Factor(s) Present in the DA-3 Cell Supernatants. The results of the preliminary treatments suggested that the inhibitory component is lipophilic, since carbohydrates, proteins, and peptides would be confined to the aqueous phase of the chloroform-methanol extraction. A lipoprotein could not be the potential inhibitory material since the solvent used is known to denature these molecules. However, it was possible that a glycolipid could be the bioactive component since it is well known that some glycolipids, such as gangliosides, are overexpressed and are shed by various tumor cells (29–31). In addition, gangliosides have been shown to be capable of altering different parameters of the immune response (32, 33). To further analyze the nature of the lipid-like inhibitory factor, the crude lipids were resuspended in chloroform and fractionated by silica gel column chromatography by sequential elution with HPLC grade chloroform, acetone, and methanol (13). Thus, nonpolar neutral lipids were eluted first, glycolipids which have a medium polarity were next eluted, and phospholipids, which are the most polar of the lipids, were eluted last. Aliquots containing 130 μg/ml were prepared from each of 5-ml fractions obtained by this separation procedure and tested as possible inhibitors in the Griess reaction using PEM from normal mice. As seen in Fig. 2, the NO_2^- inhibitory activity was confined to fractions 110 to 120, with a peak activity in fraction 114. These results indicated that the active component(s) was highly

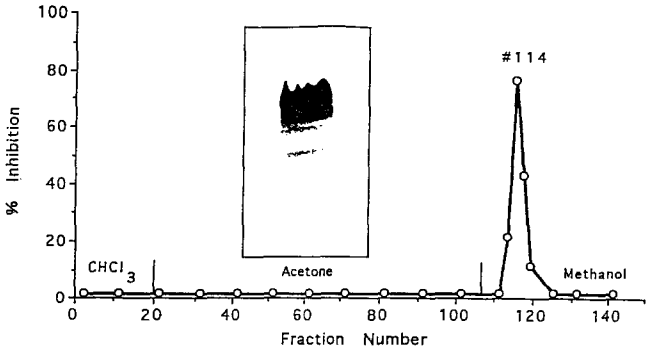

Figure 2. Preliminary fractionation of lipid extract from DA-3 cell supernatants as a function of their capacity to inhibit NO_2^- production from PEM. The crude lipids were preliminary fractionated by a silica gel column chromatography eluted as shown. 5-ml fraction were collected. PEM from normal mice (1.5×10^5/well) were pretreated with 130 μg/ml of eluted fractions for 24 h. The PEM were then washed and activated with 10 μg/ml LPS for an additional 24 h. NO_2^- levels were measured in the supernatants of the macrophage cultures as described in Materials and Methods. Values are the mean ± SE of triplicates cultures. (*Inset*) A TLC of fraction 114 developed with chloroform-methanol-acetic acid-water (65:15:10:4, vol/vol), and visualized with the molybdenum blue reagent (15).

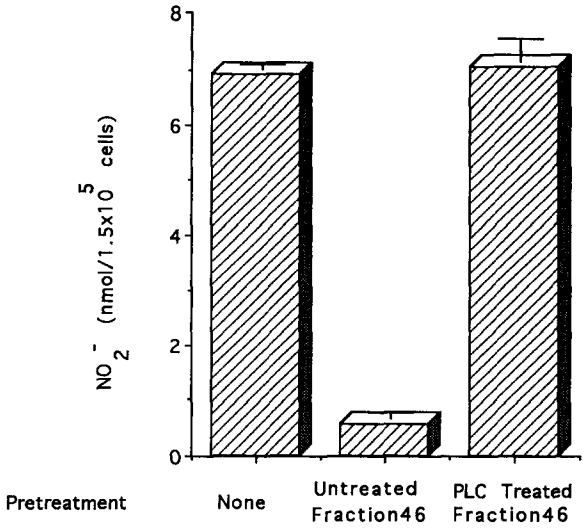

Figure 4. Effect of PLC digestion on the NO_2^- inhibitory activity of fraction 46. Fraction 46 was digested with *B. cereus* PLC, and the product was extracted with diethyl ether. The lipid extract (160 μg/ml) and the untreated fraction 46 (160 μg/ml) were assayed for NO_2^- inhibition in LPS (10 μg/ml)-activated PEM as described in Materials and Methods. Data are expressed as the mean ± SE of triplicate cultures of four different experiments.

polar, and that neutral lipids were not likely involved in the observed inhibition of NO_2^-. To further characterize the material(s) present in fraction 114, TLC was employed. After the chromatographic development was completed, the chromatogram was sprayed with either the molybdenum blue reagent which stains phospholipids (15) or with resorcinol which reacts with glycolipids (14). The absence of glycolipids in fraction 114 was demonstrated by the tar-colored reaction observed in the resorcinol assay (data not shown). A positive reaction would have been a blue-violet color. However, when the TLC was sprayed with the molybdenum blue reagent, the characteristic blue color for phospholipids was observed, suggesting that phospholipids were present in the fraction containing the bioactive property (*inset* Fig. 2).

Since fraction 114 may be comprised of more than a single component, it was further fractionated on a second silica gel column chromatography, this time eluted with mixtures of chloroform and methanol (16). This second chromatographic separation showed the highest inhibitory activity in fraction 46 (Fig. 3). When a TLC of fraction 46 was performed it gave evidence of the presence of a single molybdenum positive spot with retention factor (R_f) = 0.75 (Fig. 3, *right inset*). HPLC analysis of fraction 46 showed two peaks with retention time (R_t) of 1.81 and 3.51 min (Fig. 3, *left inset*). The bioactive analysis of these peaks demonstrated that they corresponded to chloroform and to the putative NO inhibitor, respectively (data not shown). In previous studies, Ladisch et al. (29, 32), Hakomori and collaborators (30, 34), and other investigators (31, 33, 35) have provided evidence that traces of glycolipids from tumor cells have the capacity to act on immune effector cells and alter their functions. To obtain further assurance of the phospholipid nature of the inhibitory material, a digestion of the bioactive factor with PLC was carried out.

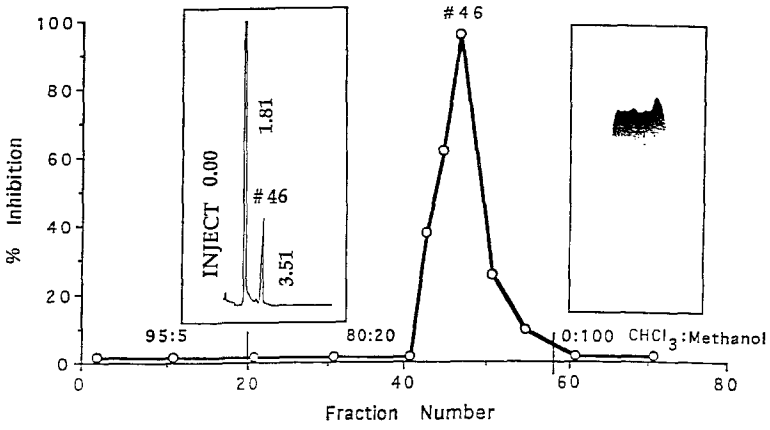

Figure 3. Isolation of a NO inhibitor from the methanolic fraction collected during the preliminary fractionation of the lipids extracted from DA-3 cell supernatants. The methanolic fraction from the previous fractionation of the crude lipids was concentrated, resuspended in chloroform-methanol (95:5), and charged on a silica gel column. The column was successively eluted with chloroform-methanol mixtures as indicated. 5-ml fractions were collected and assayed for their NO_2^- inhibitory activity on PEM as described in Materials and Methods. Values are the mean ± SE of triplicate cultures. (*Right inset*) TLC of fraction 46 developed and visualized as in Fig. 2. (*Left inset*) HPLC analysis of fraction 46 using a 3.9 mm × 30 cm μPorasil™ column with a mobile phase of acetonitrile-methanol-85% phosphoric acid (390:5:4.5, vol/vol) flowing at 2.0 ml/min. UV detection was monitored at 205 nm (17).

Digestion of Fraction 46 with PLC. Since our results suggested that the NO inhibitor is a phospholipid, we subjected the inhibitory fraction 46 to enzymatic degradation with PLC in order to provide further confirmation of the phospholipid nature of this factor (18). After the reaction mixture was shaken at room temperature for 2 h, it was extracted three times with 4-ml portions of diethyl ether. This ether extract was analyzed by TLC using hexane-diethyl ether (50:50, vol/vol) as solvent and then treated with iodine vapors to localize the degradation products from the enzymatic reaction. When a parallel TLC of the ether extract was sprayed with the molybdenum blue reagent, the blue coloration of phospholipids was not visualized, indicating that fraction 46 was completely degraded by PLC (data not shown). The products from the enzymatic digestion were scratched from the nonsprayed TLC, deabsorbed with methanol, and the methanol solution was dried under nitrogen before it was tested for inhibition of macrophage production of NO. In Fig. 4, it can be seen that the products from the digestion of fraction 46 with PLC had lost their bioactivity, confirming that a phospholipid is responsible for the nitrite inhibition caused by the supernatants of the DA-3 tumor cells cultures.

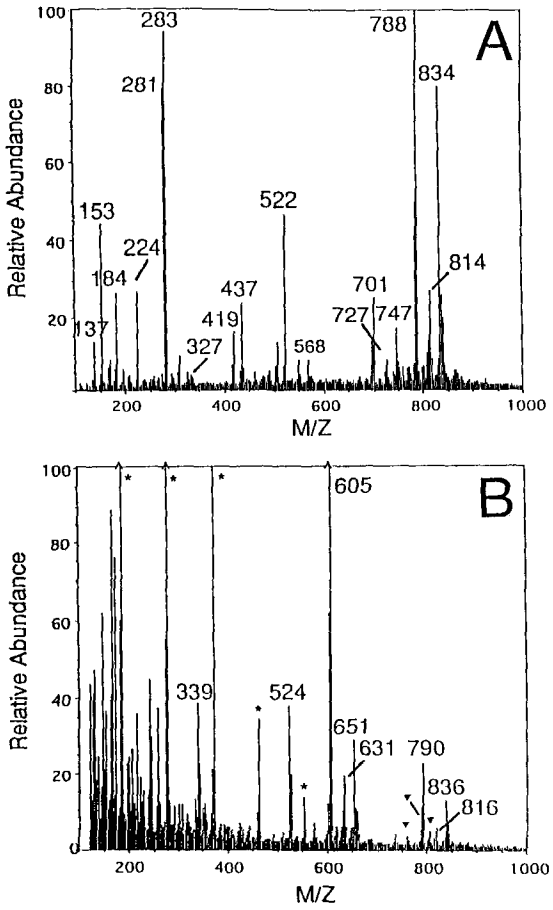

Figure 5. FAB mass spectra of the purified active fraction 46. (*A*) Negative ion mode spectrum shows peaks at m/z 788, 814, and 834 representing the $[M-H]^-$ ions of three PS molecular species with total acyl group compositions 36:1 (carbon chain length/double bonds), 38:2, and hydroxy 38:0, respectively. Corresponding fragment ions at m/z 701, 727, and 747 ($[M-H-87]^-$) are diagnostic for the loss of the serine residue from the head group. Low mass fragments at m/z 137, 153, 184, and 224 support the assignment as PS molecular species (6, 7). Carboxylate anions at m/z 281 (18:1), 283 (18:0), and 327, weak, (h 20:0) indicate that the fatty acyl groups in the two major PS molecular species are likely 18:0/18:1 (m/z 788) and 18:0/hydroxy 20:0. This conclusion was supported by product ion spectra of the $[M-H]^-$ and $[M-H-87]^-$ ions of these two molecular species (data not shown). (*B*) Positive ion mode spectrum displays $[M+H]^+$ peaks for the three PS molecular species detected in the negative mode analysis at m/z 790, 816, and 836, together with expected fragments formed by loss of the head group, $[M+H-85]^+$, at m/z 605, 631, and 651. Minor peaks representing the $[M+H]^+$ ions of traces of PC molecular species from an adjacent inactive fraction can also be seen (▼). (*) Glycerol matrix peaks.

Analysis of Fraction 46 by FAB-MS. To elucidate the molecular structure of the phospholipid responsible for the inhibition of nitrite production, fraction 46 was subjected to FAB-MS in the positive and negative modes. The results from the negative ion FAB mass spectrum of the purified active fraction 46 are shown in Fig. 5 *A*, where two predominant high mass peaks at mass-to-charge ratio (m/z) 788 and 834 and a minor peak at m/z 814 are readily apparent. Diagnostic fragments in the spectrum at m/z 137, 153, 184, 224, 701, 727, and 747 suggested that the three high mass ions represent the $[M-H]^-$ ions of three PS molecular species with total acyl group compositions of 36:1 (carbon number/double bonds) and hydroxy 38:0 and 38:2, respectively (6, 7). Carboxylate anions at m/z 281, 283, and 327 (weak) indicated that the fatty acyl groups in the two major molecular species were likely 18:0/18:1 (m/z 788) and 18:0/hydroxy 20:0 (m/z 834). This was supported by B/E linked scan (production ion) spectra of the two major $[M-H]^-$ ions and their corresponding $[M-H-87]^-$ fragments by FAB collisionally induced dissociation-tandem MS, which confirmed the proposed acyl groups compositions and further indicated that the oleoyl (18:1) and hydroxyeicosanoyl (h 20:0) groups were at the sn-2 positions (7, 36) in the two major components of PS (data not shown). GC-MS analysis after transesterification of fraction 46 with NaOMe in MeOH and subsequent trimethylsilyation of the liberated fatty acids revealed that the hydroxyl group of the hydroxy 20 carbon acyl group was in the α position (M^+ at m/z 414, M-59 at m/z 355, base peak).

The positive ion mode FAB spectrum of the fraction 46 is presented in Fig. 5 *B* (*, matrix peaks). The expected $[M+H]^+$ ions for the PS molecular species were observed, along with the corresponding diagnostic fragments for PS at $[M+H-185]^+$ (7). Minor $[M+H]^+$ peaks representing traces of PC molecular species from an adjacent inactive fraction were also observed at m/z 760, 788, and 806 (▼). PG and SM were also separated in the inactive fractions and characterized by FAB-MS. These studies strongly suggest that PS is the NO inhibitory molecule derived from the mammary tumor cells, and that the minor phospholipids, i.e., SM and PG, and the more abundant PC, are not playing a crucial role in this modulatory activity. In both the positive and negative ion mode FAB spectra of fraction 46, ions representative for glycolipids were absent. The role played by acyl groups,

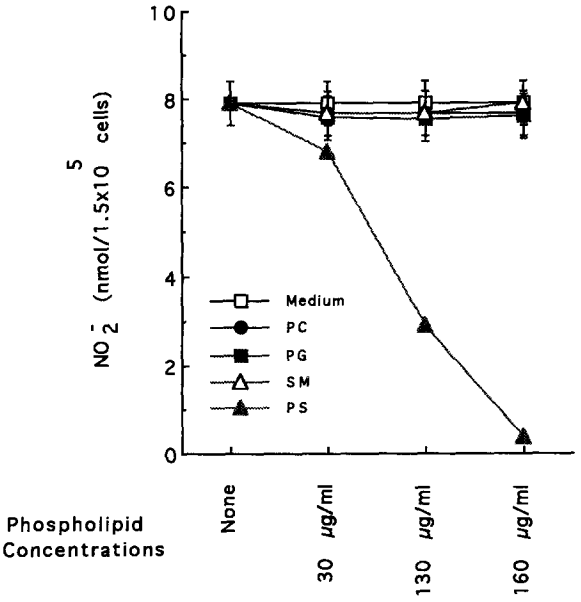

Figure 6. Effect of phosphatidyl choline (*PC*), phosphatidyl glycerol (*PG*), sphingomyelin (*SM*), and phosphatidyl serine (*PS*) on the LPS-induced macrophage production of NO_2^-. PEM were incubated for 24 h with PC, PG, SM, or PS at the concentrations indicated. After this time, the macrophages were washed and incubated for an additional 24 h with fresh medium containing LPS (10 μg/ml). The supernatants of the macrophage cultures were collected and NO_2^- was measured as described in Materials and Methods. Each value is the mean ± SE of three replicates from four different experiments.

carbons numbers, unsaturation, and the presence and/or location of hydroxyl groups at the sn1 or sn2 positions of PS is at present under investigation to determine structure–activity relationships.

To unequivocally confirm these results, we purchased commercially available PS, PC, PG, and SM. We then used these standard compounds as potential NO inhibitors in the assay for production of nitrites by PEM from normal mice. As seen in Fig. 6, PC, PG, and SM ranging from 30 to 160 μg/ml did not affect the levels of nitrite produced by the PEM from normal mice stimulated with LPS. In sharp contrast were the results obtained with PS which had a dose-dependent inhibitory activity on the production of NO by LPS-stimulated PEM, which resulted in a total abrogation of NO production at concentrations of 160 μg/ml.

Comparison between NO Production and Cytotoxicity of PEM. To ascertain that the inhibition of nitrite production brought about by the presence of PS correlates with the loss of cytotoxic potential previously seen in PEM from tumor-bearing mice, experiments were performed to concomitantly analyze these two parameters in cultures of PEM from normal mice treated with the purified fraction 46 or with PS from two commercially available sources. In our experiments we included N^GMMA as a control for the inhibition of NO production and cytotoxicity of macrophages since this is a known NO inhibitor whose actions result in the loss of cytotoxic activity by macrophages (4, 37). N^GMMA was added together with the LPS to the PEM, since it has been shown

Table 2. *Correlation of NO_2^- Production and Cytotoxicity of PEM from Normal Mice Pretreated with Various Species of PS*

Inhibitor	Dose	NO_2^-(nmol/1.5 × 10^5 cells)*	Percent cytotoxicity‡
None	–	7.64 ± 0.4	48.4 ± 2.0
	500 μM	0.12 ± 0.4	12.7 ± 2.0
N^GMMA§	50 μM	3.20 ± 0.5	30.4 ± 2.1
	5 μM	7.25 ± 0.5	46.4 ± 1.0
	160 μg/ml	0.43 ± 0.5	17.0 ± 1.1
Fraction 46‖	130 μg/ml	3.20 ± 0.2	28.3 ± 1.7
	30 μg/ml	5.47 ± 0.4	37.3 ± 1.0
	160 μg/ml	0.49 ± 0.5	18.2 ± 2.2
Bovine	130 μg/ml	3.16 ± 0.5	29.5 ± 1.1
brain PS‖	30 μg/ml	5.77 ± 0.5	38.0 ± 2.0
	160 μg/ml	0.36 ± 0.5	16.3 ± 1.5
PS (18:0/18:1)‖	130 μg/ml	2.90 ± 0.5	26.8 ± 2.1
	30 μg/ml	6.80 ± 0.3	39.6 ± 2.4

* NO_2^- levels were measured in the supernatants of the macrophage cultures as described in Materials and Methods. Values are the mean ± SE of triplicate cultures.

‡ After 24 h of stimulation with LPS, macrophages were washed twice and cocultured for 18 h with DA-3 targets in fresh medium (10^4 cells/well). Percent specific cytotoxicity was determined by the MTT method (19) as described in Materials and Methods. Values are the mean ± SE of triplicate cultures from a representative experiment.

§ PEM from normal mice (1.5 × 10^5 cells/0.2 ml) were cocultured for 24 h with the indicated concentrations of N^GMMA and LPS (10 μg/ml).

‖ PEM from normal mice (1.5 × 10^5 cells/0.2 ml) were incubated for 24 h with the indicated concentrations of the inhibitors. Fresh medium with LPS (10 μg/ml) was added to macrophage cultures for an additional 24 h.

that this inhibitor does not function unless it is cocultured with macrophages in the presence of LPS (37).

PEM were pretreated with either fraction 46, or commercial PS for 24 h, washed twice, and then stimulated with LPS (10 μg/ml) for an additional 24 h. After this time, the supernatants (100 μl) were collected and mixed with 100 μl of the Griess reagent for the quantitation of NO_2^- production by macrophages. DA-3 target cells (10^4) were added to the macrophage cultures, and cocultured with fresh medium for 18 h. Table 2 shows that the NO production by macrophages decreases in proportion with increasing concentrations of fraction 46, and that this inhibition of NO production parallels a decrease in macrophage cytotoxicity. Similar results were found when PEM were pretreated with either the crude PS extract from bovine brain or with the commercially available major molecular species of fraction 46, PS (18:0/18:1). These results strongly indicate that NO is the crucial molecule responsible for the lysis of DA-3 mammary tumor cells by macrophages. Furthermore, these data unequivocally characterize PS as the NO inhibitor produced by the DA-3 tumor cells.

Screening of Supernatants from Tumor Cell Lines for their Capacity to Inhibit NO Production/Release in Normal PEM. To investigate whether supernatants from other tumor cell types are capable of inhibiting the production and/or release of NO, we tested the supernatants of a panel of cell lines known to have various levels of PS present on their surfaces (10), i.e., A375 melanoma with 75 ± 10 ng PS/10^4 cells, the squamous cell carcinoma A431 with 39 ± 6 ng PS/10^4 cells, and the normal epidermal keratinocyte NHEK with 11 ± 2 ng PS/10^4 cells. None of the supernatants of these cell lines had NO inhibitory properties, a result that could be potentially due to the lack of secretion of PS from the cell surface into the culture medium. In addition, we tested two other murine mammary adenocarcinoma cell lines for NO inhibitory activity. Whereas no NO inhibition was obtained with the nonmetastatic 168 cells, a 10% inhibition of NO could be detected when 50% supernatants of the 410.4 metastatic mammary tumor cells were used as pretreatment of normal PEM before LPS stimulation. Studies are planned to measure actual levels of PS released in the culture medium by the different tumor cells, including the DA-3 mammary tumor cells.

Evaluation of Biological Functions of PS-treated PEM. We next examined the effects of PS on the viability and functionality of PEM, by studying a number of activities of the macrophage functional repertoire, namely phagocytosis, the reduction of MTT, superoxide production, total protein content from lysates, and the production of IL-1α and IL-6. Phagocytosis of particles by macrophages result in an increase of oxygen consumption with subsequent production of oxygen-derived radicals (38), a NADPH-utilizing pathway, as is NO production (39). Accordingly, when PEM were pretreated with PS and then activated with LPS, they phagocytosed 25 ± 5 zymosan particles/cell, whereas non-

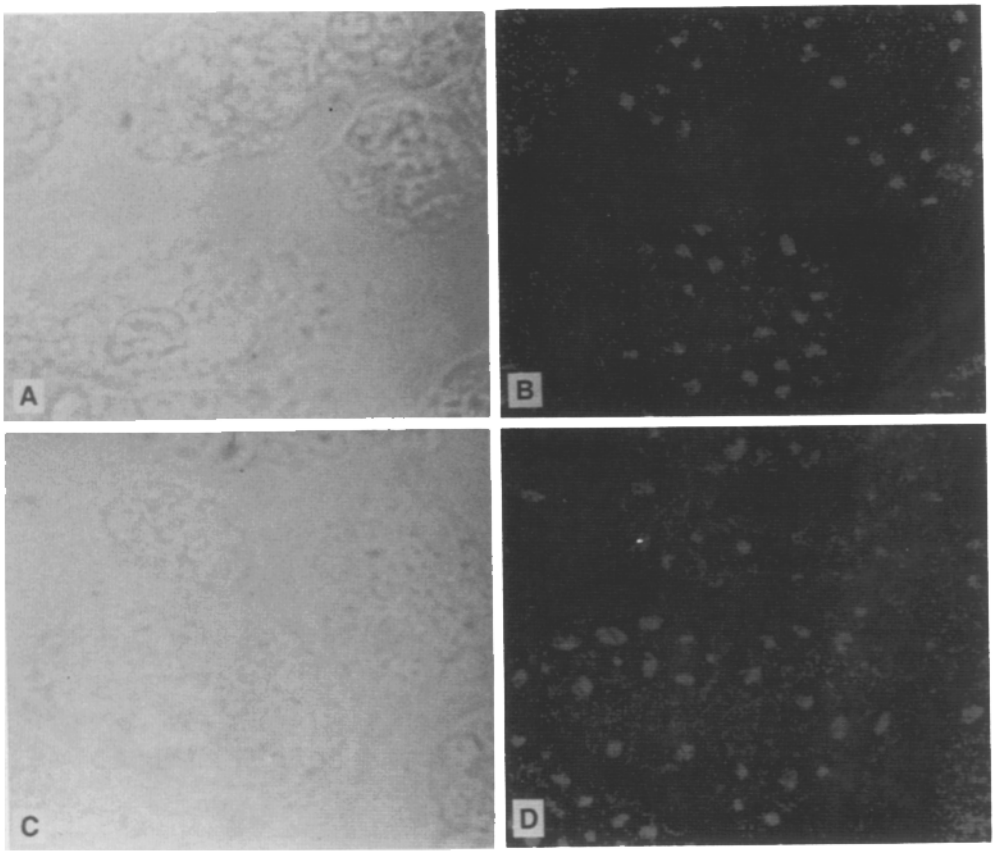

Figure 7. Phagocytosis of zymosan particles by PS-treated PEM. Macrophages were nontreated (*A*) or treated (*C*) with PS, stimulated with 10 μg/ml LPS, and cultured with zymosan particles as indicated in Materials and Methods. The cells were then examined on an inverted Nikon microscope equipped with a laser-scanning device. Transmitted light images from the laser-scanning confocal microscope (*A* and *C*) are paired with the corresponding refracted light image of the same field (*B* and *D*). Images are of 0.6-μm-thick optical section.

PS–pretreated PEM gave an average of 19 ± 5 phagocytosed particles/cell. A microscope observation of the phagocytic activity of PS-pretreated PEM can be seen in Fig. 7, where the confocal laser scanning image produced with refracted light demonstrates the presence of slightly higher numbers of particles within these cells as compared to the particles ingested by untreated PEM. These results correlate with the slightly higher O_2^- production by PS-pretreatment PEM as compared to the levels produced by the untreated control PEM (Table 3). These data indicate the integrity of the NADPH-O_2 system in PS-pretreated PEM. Likewise, PS-treated PEM preserve their capacity to reduce MTT, which reflects the optimal response of the mitochondrial dehydrogenases (19). Additional evaluations showed that the level of total protein concentration of adherent cells was not affected in PS-pretreated, LPS-activated PEM as compared with the untreated control (Table 3). Similarly, the amounts of IL-1α and IL-6 produced by untreated and experimental PEM did not show significant differences. Thus, the results presented in Table 3 demonstrate that the viability and functionality of PS-pretreated PEM has not been impaired by treatment with the phospholipid.

Effects of PS on the Production of NO by PEM Activted with Nonlipid Stimulators. To rule out the possible lipid–lipid interaction of PS and LPS as a possible cause of the downregulation of NO production by PS-pretreated PEM, we examined the effect of nonlipid stimulators of macrophages as inducers of NO production. As shown in Table 4, 160 μg/ml PS virtually abolished the capacity of PEM to produce NO upon activation with either LPS (10 μg/ml), IFN-γ (500 U/ml), or SEB (300 μg/ml). These results suggest that the inhibitory activity of PS on PEM is not due to a direct interaction between two molecules of lipid nature since the same results were obtained when nonlipid stimulators were used.

Kinetics of the Inhibitory Activity of PS on LPS-activated PEM. In Fig. 8, the kinetics of the NO inhibitory activity of PS on PEM is depicted, illustrating that at least 2 h of PS pretreatment are needed to visualize a decrease of NO production by LPS-activated PEM. Preincubation of PEM with PS for 2, 4, 6, or 8 h resulted in a profound inhibition of their potential to produce/release NO, and this was further accentuated in PEM cultures that were pretreated with PS for 18 h. Addition of PS to macrophage cultures at the

Table 3. *Evaluation of Viability and Intact Functionality of PS-treated PEM*

Parameter assayed	Untreated PEM	PS-treated PEM
O_2^- production*		
(nmol/1.5 × 10^5 cells)	1.55 ± 0.03	1.89 ± 0.03
Mitochondrial‡		
dehydrogenases (OD)	0.820 ± 0.03	0.911 ± 0.07
Total protein levels§		
(μg/ml)	501 ± 46	497 ± 44
IL-1-α(pg/ml)‖	300 ± 66	375 ± 15
IL-6 (pg/ml)‖	18,285 ± 3,000	18,309 ± 3,805

PEM from normal mice (1.5 × 10^5 cells/0.2 ml) were cultured for 24 h by themselves or in the presence of 160 μg/ml PS, washed with RPMI-1640, and then activated with 10 μg/ml LPS as indicated in Materials and Methods. All experiments are the mean ± SE of triplicate cultures from three representative experiments.

* Superoxide (O_2^-) production was assayed by the method of Pick and Mizel (20). Experimental PEM were incubated for 1 h with 100 μl of 160 μM ferricytochrome c in HBSS. The absorbance was measured at 550 nm.

‡ The optical density of formazan salt produced as a consequence of MTT reduction (19), was measured at 570 nm.

§ The protein assay was performed following the method of Lowry et al. (21).

‖ IL-1-α and IL-6 were measured with commercially available ELISA kits.

Table 4. *Effects of PS on the Production of NO_2^- by PEM Activated with LPS and Nonlipid Stimulators*

Activator	Control PEM	PS-treated PEM
LPS (10 μg/ml)	7.64 ± 0.2	0.36 ± 0.1
IFN-γ (500 U/ml)	0.7 ± 0.1	0.06 ± 0.01
SEB (300 μg/ml)	0.52 ± 0.1	0.04 ± 0.01

Normal PEM (1.5 × 10^5 cells/0.2 ml) were cultured with or without (*control PEM*) 160 μg/ml PS for 24 h. The cells were then washed and cultured with fresh medium supplemented with activators as shown above for a period of 18 h. Nitrite was measured as described in Materials and Methods. Values are the mean ± SE of triplicate cultures from three representative experiments.

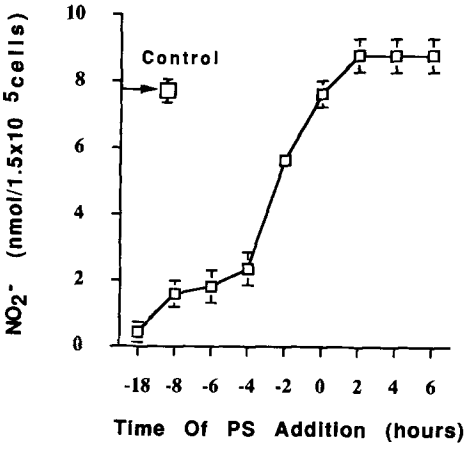

Figure 8. Effect of time of PS addition on the LPS activation of PEM to produce NO. PS (160 μg/ml) was added to macrophage cultures (1.5 × 10^5/0.2 ml) before (*negative numbers*), at the same time (*zero*), or after (*positive numbers*) activation with 10 μg/ml LPS as indicated. After 18 h of activation, NO_2^- was measured as described in Materials and Methods. Only cultures preincubated with PS (*negative numbers*) were washed with RPMI before the addition of LPS. The values are the mean ± SE of triplicate cultures from three independent experiments.

time of stimulation (zero time) did not interfere with their normal production/release of NO. Treatment of PEM with PS after 2, 4, or 6 h of activation with LPS, produced a small increase in the production of NO. Therefore, a pretreatment appears to be required in order to observe the inhibitory activity of PS in the production and/or release of NO.

Reversibility of the Inhibition of NO Production by PS-pretreated PEM. As previously shown, maximal NO inhibitory activity of PS (95%) is observed when PEM are pretreated with PS for 24 h, washed with RPMI-1640, and immediately incubated for 18 h with medium containing 10 μg/ml LPS. However, if the addition of LPS to macrophage cultures is delayed for a period of 24 or 48 h after removal of PS, the inhibition of NO declines to 67 and 38%, respectively (Fig. 9). These results indicate the partial reversibility of the inhibitory activity of PS and further substantiate the intact viability and functionality of PEM pretreated with PS.

iNOS Activity. In further studies we evaluated whether PS could affect the activity of iNOS. PEM were incubated for 24 h with medium supplemented or not (control) with 160 μg/ml PS. After completion of the incubation time, the macrophage layers were washed and cultured for an additional 18 h with medium containing 10 μg/ml LPS. The iNOS activity of PEM cytosols (200 μg of protein) in the presence of defined cofactors (FAD, NADPH, BH_4 and DTT) was determined. iNOS activity was not detectable in the cytosol of macrophages preincubated with PS (Table 5). However, control macrophages, not preincubated with PS, showed 10.66 ± 0.3 nmol of NO_2^- as iNOS activity. Table 5 also illustrates that if before the addition of L-arginine to the reaction mixture, the iNOS-active cytosol is preincubated for 30 min with either 5–20 μg PS or 10–30 μg of the cytosol of LPS-activated PEM pretreated with PS, the capacity of the active cytosol to produce NO remains unaffected. Preincubations of 10 and 20 min gave similar results (data not shown). These data suggest that PS could be blocking the induction of iNOS.

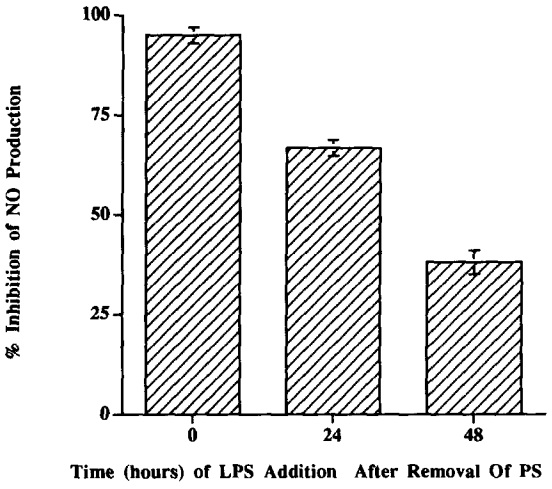

Figure 9. Reversibility of the inhibition of NO production by PS-treated PEM. Normal PEM (1.5×10^5/0.2 ml) were cultured for 24 h with or without (control) 160 μg/ml PS. The cell monolayers were washed twice with RPMI, added fresh culture medium, and activated for 18 h with 10 μg/ml LPS. The addition of LPS to cell cultures was done either immediately (zero time) or delayed for 24 or 48 h. NO_2^- in cell cultures was measured as described in Materials and Methods. Data are expressed as the mean ± SE of triplicate cultures from three representative experiments.

Table 5. *Effects of PS and the Cytosol of Activated PEM Pretreated with PS on iNOS Activity*

Cytosol from activated PEM	Cytosol from activated PEM pretreated with PS	PS	NO_2^-
μg	μg	μg	nmol
200	–	–	10.66 ± 0.3
–	200	–	0.09 ± 0.2
200	10	–	11.12 ± 0.5
200	20	–	9.57 ± 0.5
200	30	–	8.50 ± 0.2
200	–	5	9.00 ± 0.5
200	–	10	9.20 ± 0.5
200	–	20	9.40 ± 0.5

LPS-activated macrophage cytosol (200 μg) from normal PEM treated or untreated with PS were assayed for iNOS activity in the presence of defined cofactors as described in Materials and Methods. In some wells, cytosol from LPS-activated normal PEM (200 μg) was incubated for 30 min either with PS or cytosol from activated PEM pretreated with PS before the addition of L-arginine to the reaction mixture. Nitrite levels were normalized according to the protein concentration of each experimental assay. Values are the mean ± SE of duplicate cultures from three independent experiments.

Discussion

Activated macrophages play an important role in host-defense mechanisms (40, 41), against cancer (42), and against infections (43). The acquisition of the activated state is a complex process that can be achieved under appropriate stimulation of macrophages with naturally occurring substances or with synthetic agents (44). LPS is a well-known activating agent that can trigger the macrophage cytotoxicity on tumor targets either alone or in combination with lymphokines (45, 46). Although tumor cells are heterogeneous with regard to many characteristics (47), they seem to share susceptibility to destruction by activated macrophages. In this regard, Hibbs et al. (4, 23, 48) have shown that the crucial agent that directly contributes to the tumoricidal activity of macrophages is NO, a freely diffusible, short-lived, and highly toxic molecule. Similar results reported by other investigators (12, 49) support this concept. Therefore, the integrity of the circuit of macrophage activation to kill tumor cells must be maintained in order to prevent the subversion of the host immunity by tumors. However, during neoplasm development, tumor cells either directly or indirectly appear to impair one or more steps of this complex machinery as a mechanism to evade their destruction by activated macrophages (1, 50). In the present

study, we provide evidence that tumor-derived PS could be one of such immune regulatory agents with the capacity to protect tumor cells from destruction via the impairment of NO production by macrophages.

Various reports have demonstrated that PS possesses immunomodulating properties in vivo or in vitro, including the enhancement of the ability of epidermal Langerhans cells to induce contact hypersensitivity (51), the augmentation of the binding of peptides to purified class II MHC molecules (52), and the inhibition of DNA synthesis in lymphocytes (53). Furthermore, and more pertinent to our studies, PS has been found to inhibit the lymphokine-induced macrophage microbicidal activity against *Leishmania major* (54). Thus, we could speculate that this inhibition of the microbicidal activity of the macrophages could be mediated by the NO-inhibitory property of PS. This conjecture is supported by the publications of Liew et al. (55) and Green et al. (56) who coincide in pointing out that the production of NO is a crucial event in macrophage killing of *L. major*. As a whole, these observations suggest that the inhibition of NO production in macrophages by PS represent an important regulatory mechanism applicable to situations where macrophage cytotoxicity is of relevance.

During the last 20 yr, compelling evidence has been presented that lipids of biological membranes are asymmetrically distributed, providing in this way, a molecular basis for their functional diversity (57, 58). This functional asymmetry is best exemplified by the frequent preservation of PS within the inner leaflet of cells (59, 60). Many investigators have shown that failure of cells to keep this rule is usually associated with their recognition by mononuclear phagocytes, as is the case with sickled cells or resealed red cell ghosts (61–64). Recently, the asymmetric distribution of PS in cell membranes is gaining increasing attention. The report of Utsugi et al. (10) is of particular interest. These authors noted that when activated human blood monocytes bind to tumor cells that overexpress PS in their outer membrane leaflet, target cell destruction ensues. In contrast, normal keratinocytes which externally express PS in comparatively lower amounts than those in tumor cells, are not bound or lysed by the monocytes. In a recent review, Savill et al. (65) have suggested the presence of at least three classes of receptors in phagocytes for the recognition of apoptotic cells, which include receptors for lectin, thrombospondin, and PS. These observations, together with our findings, suggest that tumor cells that express PS in their outer leaflet could be targeted to be lysed by macrophages. However, the overexpression and shedding of PS by the tumor cells could result in the blockage of the putative PS receptors present in the macrophages. By altering this tightly controlled process, tumor cells may evade the host defenses provided by the macrophages. The ubiquity of PS in cell membranes from different tissues, together with its NO-inhibitory property, indicate that this phospholipid may be one of the long sought-after and hitherto uncharacterized molecules responsible for regulating the physiological levels of NO.

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