Liposomal Induction of a Heat-stable Macrophage Priming Factor to Induce Nitric Oxide in Response to LPS

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Purpose. The effects of liposomes on nitric oxide (NO) production from mouse peritoneal macrophages following intraperitoneal injection of liposomes were investigated.

Methods. Mouse peritoneal macrophages were collected following intraperitoneal injection of liposomes and cultured with and without lipopolysaccharide (LPS). Peritoneal washing fluid was also collected from the mice injected with liposomes. NO production was evaluated by measuring the concentration of nitrite in the macrophage culture supernatant by Griess reagent.

Results. NO production stimulated by LPS was observed in peritoneal macrophages obtained from the liposome-treated mice, but liposomes did not activate macrophages directly to induce NO in response to LPS. NO production was higher in the liposomes composed of phosphatidylcholine than that of negatively charged liposomes composed of phosphatidylserine. Peritoneal washing fluid obtained from mice injected with liposomes has a capacity to induce NO production in the macrophages from naive mice. This capacity was not diminished by heat-treatment at 100°C for 5 min.

Conclusions. Peritoneal macrophages were activated to produce NO in response to LPS following intraperitoneal injection of liposomes. They did not activate macrophages directly, and the induction of heat-stable macrophage priming factor, but not cytokines, is suggested.

KEY WORDS: liposomes; macrophages; nitric oxide; heat-stable macrophage priming factor.

INTRODUCTION

Nitric oxide (NO), a reactive free-radical gas, was found to be generated enzymatically from L-arginine and molecular oxygen by constitutive or inducible NO synthase (NOS) in various cells such as neuronal cells, endothelium, adrenalglands, neutrophils, Kupffer cells, and activated macrophages (1). In activated macrophages, microbicidal and tumoricidal capacity is mainly due to the ability to produce NO. In the activation of macrophages for cytotoxic effector cells, a two-stage reaction process is known as priming and triggering, and NO production has not been observed when resident macrophages were stimulated by IFN-γ or LPS alone (2). In macrophage NO generation by inducible NOS (iNOS), interferon-γ (IFN-γ) is widely recognized as a major priming factor, and some other cytokines or lipopolysaccharide (LPS) are required to trigger the full induction of NO from activated macrophages (3).

Liposomes are phospholipid vesicles that have been proposed as biodegradable carriers of drugs and DNA. To date, liposomes have been shown very useful medically for cases in which the desired sites of drug delivery involve macrophages. Negatively charged liposomes containing phosphatidylserine (PS) or phosphatidic acid (PA) are preferentially taken up by phagocytic cells such as macrophages in a receptor-mediated manner (4). Consequently, liposomes have been studied extensively for their potential as carriers of antigens to macrophages or immuno adjuvants (5). The effects of liposomes on NO production in macrophages, however, have been given but little attention. Examination was made of the effects of liposomes on NO production from mouse peritoneal macrophages following intraperitoneal injection and liposomes composed of phosphatidylcholine activated macrophages to produce NO in response to LPS stimulation.

METHODS

Mice

C3H/HeN mice (6–8 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan), and kept under specific pathogen-free conditions with food and water *ad libitum*. Animal use and relevant experimental procedures were approved by the Tokyo University of Pharmacy and Life Science Committee on Care and Use of Laboratory Animals.

Chemicals and Reagents

Phosphatidylcholine (PC) from egg yolk was kindly provided by Nippon Oil and Fat Co., Ltd. (Tokyo, Japan). Lipopolysaccharide (LPS) from *Escherichia coli* (serotype 0111:B4), and PS from calf brain were purchased from Sigma (St. Louis, MO, USA).

Preparation of Liposomes

Multilamellar liposomes composed of phosphatidyl choline:cholesterol (1:1, PC-liposomes) and phosphatidylcholine:phosphatidylserine:cholesterol (1:1:2, PS-liposome) were prepared by a voltexing method and then passed through a membrane filter (0.45 μm; Corning Glassworks, Corning, NY) before use. The yield of liposomes was ca. 85%. LPS contamination in the liposome preparation and culture media was routinely assessed using a Limulus amoebocyte lysate assay (Wako Pure Chemicals Co., Ltd., Osaka, Japan), and the concentration of LPS in any liposomal preparation was less than 18 pg/ml.

Preparation of Macrophages

Mice were injected with 1.0 ml liposomes (4 μmol lipid/mouse). Peritoneal exudate cells (PEC) were obtained by peritoneal lavage with 10 ml ice-cold Hanks balanced salt solution (HBSS, Ca^{2+} and Mg^{2+} free) supplemented with 10 U/ml heparin. PEC were washed twice and resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum and overlaid on a plastic culture dish (96-well culture plate, Corning). The plates were incubated in humidified 5% CO_2 at 37°C for 2 hr to allow macrophage adherence. Each plate was washed with warmed RPMI-1640 under gentle agitation to dislodge non-adhering cells and the macrophage monolayer was then prepared. About 95% of the adhering cells were judged to be

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macrophages based on the extent of latex particle ingestion and Giemsa staining.

Nitrite Determination

Macrophages (1×10^5 cells/well of 96-well culture plate) prepared from mice intraperitoneally injected with liposomes were incubated for 48 hr with or without LPS (10 μg/ml) to elicit NO production. The amount of NO production was determined based on nitrite content in the culture supernatant using the Griess reagent as described by Stuehr and Nathan (6). In brief, 100 μl culture supernatants were mixed with 100 μl Griess reagent (1% sulfanilamide, 0.1% N-[1-naphthyl]-ethylenediamine dihydrochloride in 2% H_3PO_4) and incubated at room temperature for 10 min. Absorbance at 550 nm was measured on a microplate reader (Corona MTP-32, Tokyo, Japan). NO_2-levels were determined using $NaNO_2$ as the standard.

Preparation of Peritoneal Washing Fluid

Mouse peritoneal cavity was washed with 2.0 ml HBSS solution at 3, 6, 9, and 12 hr after intraperitoneal injection of liposomes (4 μmol lipid/mouse), and peritoneal washing HBSS were collected with a syringe and centrifuged at 2000 rpm for 10 min (Hitachi, CR5B2). The supernatant was designated as peritoneal washing fluid (PWF). One hundred microliters of PWF were added to the macrophage monolayer (1×10^5 cells/well) obtained from naive mice and incubated for 24 hr on a 96 well culture plate (Corning). The macrophages were then incubated for 48 hr with LPS (10 μg/ml) to evaluate NO production.

RESULTS

Effects of Liposomes on NO Production

NO production was estimated in peritoneal macrophages from C3H/HeN mice following intraperitoneal injection of liposomes. As shown in Fig. 1, nitrite production stimulated by LPS was observed in macrophages from PC-liposome-treated mice. There was no production of NO in macrophages stimulated without LPS.

Negatively charged liposomes composed of phosphatidylserine are preferentially taken up by phagocytic cells such as macrophages, and this is mediated by scavenger receptors (7). Examination was thus made of the effects of liposomal charge on NO production using neutral PC-and negatively charged PS-liposomes. Macrophages were activated by both to produce nitrite in response to LPS, but production in PC-liposomes was twice that in PS-liposomes (Fig. 1). By the addition of N-monomethylarginine (NMMA), a specific inhibitor of NO synthase, nitrite production from PC-liposome-treated macrophages stimulated with LPS was reduced to that of the control (Fig. 1). Thus, increase in nitrite following liposome treatment may be due to NO produced by NO synthase. It is evident from these findings that liposomes activate macrophages to induce NO in response to LPS.

Kinetics

Peritoneal macrophages were prepared from C3H/HeN mice on a specified day following intraperitoneal injection of PC- or PS-liposomes and incubated with LPS for 48 hr for assessment of NO production. As shown in Fig. 2, the profiles of NO production were essentially the same for PC- and PS-liposome treatment, and NO production was greatest in macrophages obtained on day one. NO production induced by PC-liposomes was twice that of PS-liposomes.

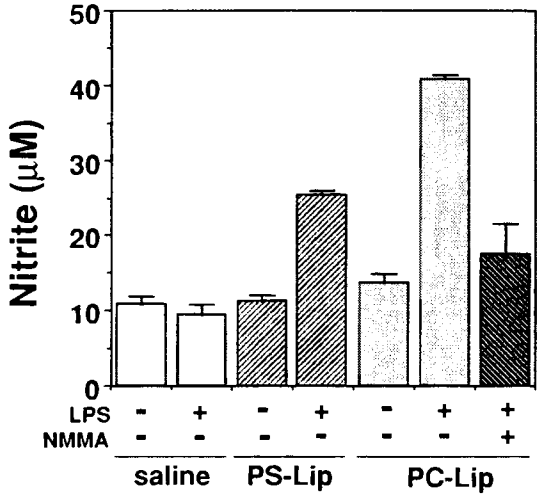

Fig. 1. Effects of liposomal charge on nitrit production in peritoneal macrophages obtained from C3H/HeN mice. Peritoneal macrophages were prepared from mice at 24 hr following intraperitoneal injection of PC- and PS-liposomes (4 μmol lipid/mouse), and then incubated without or with LPS (10 μg/ml) for 48 hr to elicit the production of nitrit. The concentration of N-monomethylarginine (NMMA) was 10 μg/ml. The values are the mean ± SD of triplicate cultures from three independent experiments.

NO Production by Peritoneal Washing Fluid

In *in vitro* experiments, NO production was not observed even on treating macrophages from naive mice with liposomes and LPS (Fig. 3). Liposomes thus do not activate macrophages directly to induce NO in response to LPS, and following liposome injection, possibly a factor which activates macrophages is secreted. To identify such a factor, peritoneal washing fluid

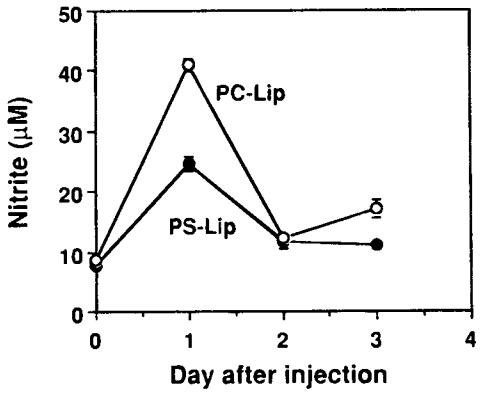

Fig. 2. Profiles of NO production following intraperitoneal injection of liposomes. Peritoneal macrophages were prepared at indicated day following liposomal injection, and then incubated without or with LPS (10 μg/ml) for 48 hr to elicit the production of NO. The values are the mean ± SD of triplicate cultures from three independent experiments.

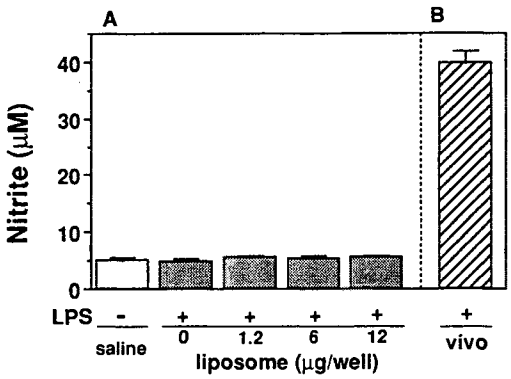

Fig. 3. Effect of liposomes on NO production in macrophages obtained from naive mice *in vitro*. (A) Peritoneal macrophages (1×10^5 cells/well) were incubated with PC-liposomes and LPS (10 μg/ml) for 48 hr, and NO production was determined by Greiss reagent. (B) Peritoneal macrophages were prepared from mice at 24 hr following intraperitoneal injection of PC-liposomes (4 μmol lipid/mouse), and then incubated with LPS (10 μg/ml) for 48 hr to elicit the production of nitrit. The values are the mean ± SD of triplicate cultures from three independent experiments.

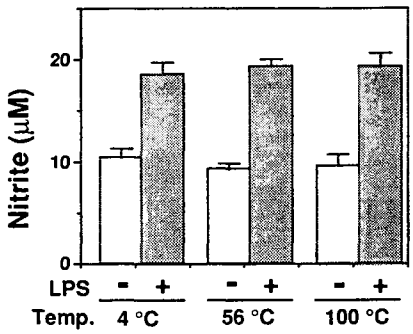

Fig. 5. Heat stability of PWF. PWF was treated with indicated temperature and NO production from macrophage obtained from naive mice was examined. Macrophages were incubated with PWF and heat-treated PWF for 24 hr, and further incubated with LPS (10 μg/ml) to elicit the production of NO. The values are the mean ± SD of triplicate cultures from three independent experiments.

(designated as PWF) following liposome injection was collected from the peritoneal cavity and the macrophage activation capacity of PWF was determined using peritoneal macrophages from naive mice. PWF prepared at 6 hr after liposomal injection activated macrophages the most and produced NO in response to LPS (Fig. 4).

This capacity was not diminished by heat-treatment at 56°C (30min) or 100°C (5 min), thus indicating a heat stable macrophage activating factor to be secreted (Fig. 5).

DISCUSSION

The microbicidal and tumoricidal capacity of activated mouse macrophages is mainly due to their ability to produce NO, and activation corresponds to the induction of a NO synthase (iNOS) which produces NO from L-arginine (3). The induction of iNOS activity in mouse peritoneal macrophages may require two signals delivered in sequence: IFN-γ is an essential for priming macrophages, and inflammatory cytokines or microbial products trigger iNOS expression in primed macrophages (3,8). However, the effects of liposomes on NO production in macrophages have not been studied in detail and thus, this was the purpose of the present study.

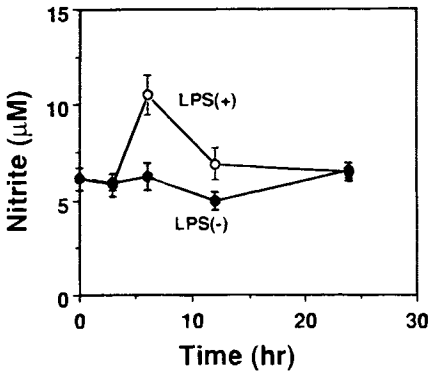

Fig. 4. NO production from macrophages by peritoneal washing fluid. Following intraperitoneal injection of PC-liposomes, mouse peritoneal washing fluid (PWF) was collected at indicated time, and the effect of PWF on NO production from macrophage obtained from naive mice was examined. Macrophages were incubated with PWF for 24 hr, and further incubated without or with LPS (10 μg/ml) to elicit the production of NO. The values are the mean ± SD of triplicate cultures from three independent experiments.

Intraperitoneally administered liposomes were clearly shown to activate macrophages to induce NO in response to LPS, whereas, in the absence of LPS, no NO production was observed even with the addition of liposomes (Fig. 1). Further, no NO production occurred when liposomes and/or LPS were added to macrophages obtained from naive mice (Fig. 3), suggesting liposomes not to activate macrophages directly to produce NO, and intraperitoneally injected liposomes to induce the secretion of a macrophage priming factor. Schroitt and Fidler (4) and Sambrano and Steinberg (7) reported negatively charged liposomes composed of PS to be preferentially taken up by phagocytic cells such as macrophages in a receptor-mediated manner. But, NO production from PS-liposome-treated macrophages was less than that of PC-liposomes (Fig. 1). NO production induced by liposomes thus does not depend on liposomal uptake by macrophages. LPS-induced NO production *in vitro* from thiglycollate exuding mouse peritoneal macrophages was previously shown to be inhibited by negatively charged liposomes, PS- and PA-liposomes, with consequent suppression of iNOS induction (9). Thus, the lower NO production in PS-liposomes may reflect inhibition of iNOS induction by PS-liposomes taken up by macrophages.

IFN-γ is an important factor for the priming of macrophages to induce NO production in response to LPS (3). Peritoneally injected liposomes induce IFN-γ production from mouse splenic cells, but not IL-4, according to our previous study (10). The induction of IFN-γ following intraperitoneal injection of liposomes may thus be considered to prime macrophages to induce NO in response to LPS. For confirmation of this, reconstitution experiments were carried out. Following the peritoneal injection of liposomes, the peritoneal cavity was washed with HBSS and PWF was collected. The macrophage activating capacity of PWF was assessed using macrophages from naive mice and PWF collected 6 hr after liposome injection showed the highest NO production in response to LPS (Fig. 4). The

production and secretion of cytokines by mitogen-activated lymphocytes require more than 24 hr (11). In this study, PWF still had macrophage priming activity even following heat treatment of PWF at 100°C at 5 min (Fig. 5). These findings suggest that a heat-stable macrophage priming factor(s) is a nonproteinic molecule(s), most probably lipid or carbohydrate in nature.

At an early stage of inflammatory events, neutrophiles which are one of phagocytic cells rapidly infiltrate into the inflammatory site and produce many substances such as platelet-activating factor (PAF), lysophosphatidylcholine and arachidonic acid (12,13). These substances affect NO production from macrophages and endotherial cells (14,15). A heat-stable macrophage priming factor would thus be produced by interaction of neutrophiles with liposomes injected intraperitonealy.

In conclusion, liposomes activate macrophages to induce NO in response to LPS stimulation. They do not activate macrophages directly and the induction of a heat stable macrophage priming factor, but not cytokines, is suggested. Study is now being made to identify this factor.

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